AFRL-RZ-WP-TR-2010-2002

THE EFFECT OF DIETHYLENE GLYCOL MONOMETHYL ETHER (DiEGME) ON MICROBIAL CONTAMINATION OF JET FUEL: A MINIMUM CONCENTRATION STUDY

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Power Division

MARCH 2010
Interim Report

AIR FORCE RESEARCH LABORATORY
PROPULSION DIRECTORATE
WRIGHT-PATTERSON AIR FORCE BASE, OH 45433-7251
AIR FORCE MATERIEL COMMAND
UNITED STATES AIR FORCE

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
March 2010	Interim	01 August 2007 – 01 August 2009

4. TITLE AND SUBTITLE
THE EFFECT OF DIETHYLENE GLYCOL MONOMETHYL ETHER (DiEGME) ON MICROBIAL CONTAMINATION OF JET FUEL: A MINIMUM CONCENTRATION STUDY

5a. CONTRACT NUMBER
F33615-03-2-2347

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
62203F

6. AUTHOR(S)
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Ellen M. Strobel (AFRL/RZPF)

5d. PROJECT NUMBER
3048

5e. TASK NUMBER
04

5f. WORK UNIT NUMBER
304804AK

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Air Force Materiel Command, United States Air Force

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Air Force Research Laboratory
Propulsion Directorate
Wright-Patterson Air Force Base, OH 45433-7251
Air Force Materiel Command
United States Air Force

10. SPONSORING/MONITORING AGENCY ACRONYM(S)
AFRL/RZPF

11. SPONSORING/MONITORING AGENCY REPORT NUMBER(S)
AFRL-RZ-WP-TR-2010-2002

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES
PAO Case Number: 88ABW-2009-3880, Clearance Date: 09 September 2009. Report contains color.

This study was undertaken as part of the minimum FSII (fuel system icing inhibitor) study effort. The funding was provided by the Defense Supply Center Richmond (DSCR) through the Air Force Petroleum Office (AFPET).

14. ABSTRACT
Diethylene glycol monomethyl ether (DiEGME) is widely used in military aircraft to inhibit both fuel system icing and microbial growth. However, due to the cost of this additive and its deleterious effects of the additive, a study is being conducted to determine if it is possible to reduce the concentration of DiEGME in military aircraft fuel. The study presented here was conducted to support the larger study investigating the possible impact of lowering the DiEGME level from ~0.05-0.15% to ~0.00-0.05% by volume in the fuel phase. The study presented here was confined to the impact upon the biocidal/biostatic properties of the additive. This study suggests that a minimum of ~0.01-0.02% DiEGME is needed to retain biocidal/biostatic effectiveness. *See Alternate Abstract on reverse →*

15. SUBJECT TERMS
bioburden, aviation fuel, bacteria, microbial contaminants, DiEGME, FSII

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT:	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON (Monitor)
a. REPORT	b. ABSTRACT	c. THIS PAGE			Ellen Strobel
Unclassified	Unclassified	Unclassified	SAR	50	19b. TELEPHONE NUMBER *(Include Area Code)* N/A

14. ABSTRACT (alternate)

Diethylene glycol monomethyl ether (DiEGME) is widely used in military aircraft as a fuel system icing inhibitor (FSII). It also has the secondary effect of acting as an inhibitor of microbial growth. However, DiEGME's high dosage rate results in significant expense for the Air Force, and DiEGME has also been implicated in a number of aircraft system problems, including tank topcoat peeling in the B-52. As a result, a large investigation is underway to determine if it is possible to reduce the amount of DiEGME in aviation fuel. The investigation utilizes a Small-Scale Icing Simulator (SSIS) to examine fuel freezing behavior by differential pressure drop evaluation and flow rate change as it varies according to freezing temperature and DiEGME concentration. It also uses information provided by Boeing studies with a B-52 strainer housing, and an investigation into the effects of alternate filter types, flow passage sizes, overall flow rate, and total filtration surface areas. Thus far, DiEGME added at a minimum of 0.04 volume % to aviation fuel appears to prevent freezing. However, none of these experiments provide information on the amount of DiEGME needed for the continued suppression of microbial growth. In order to bridge this gap, the study presented here addresses the biological component of the overall investigation. The conclusions of this study provide essential information on appropriate reduction of the current dosage of DiEGME for purposes of decreasing cost and reduction of harmful side effects, while still retaining the additive's valuable biocidal/biostatic properties. Basic questions addressed in this study include: whether DiEGME currently has any effect on microbes, what concentration of DiEGME is required to significantly affect their growth if there is an effect, and whether microorganisms recently gathered from the field may exhibit a different response to DiEGME than lab cultured microorganisms. Methodologies utilized here are primarily based on traditional culture methods. Fuel/water mixtures in French square bottles are used to simulate tank conditions. Microorganisms obtained from the American Type Culture Collection (ATCC) and from the field were introduced into these test setups, where they were challenged by DiEGME concentrations from 0-30% by volume in the water phase at ambient temperature (~0.00-0.05% by volume in the fuel phase). An additional study was also performed for the field microbes only at DiEGME concentrations of 30-60% by volume in the aqueous phase at ambient temperature (~0.05-0.15% by volume in the fuel phase). DiEGME concentrations of 30-60% by volume in the aqueous phase represent DiEGME levels that may be found currently in aviation fuel systems at ambient temperature. The 0-30% levels tested represent possible reduced concentration levels under consideration, with the goal being to discover the lowest level at which the additive is still effective at controlling microbial growth.

Results suggest that the ability of DiEGME to halt microbial growth is both concentration and microbe dependent. Concentrations greater than 10% DiEGME by volume in the aqueous phase at ambient temperature (equivalent of ~ 0.01-0.02% by volume in the fuel) were shown to have a beneficial biocidal/biostatic effect in all test cases.

TABLE OF CONTENTS

Section Page

LIST OF FIGURES

LIST OF TABLES

PREFACE

The results presented in this report are part of a larger minimum FSII study being conducted by the Air Force Research Laboratory Fuels Branch, in coordination with the Air Force Petroleum Agency. The larger study was launched in 2005 and is entitled, Investigation of the Effectiveness of DiEGME to Suppress the Solidification of Water in Aircraft Fuel Systems. Funding for both studies was provided by the Defense Supply Center Richmond (DSCR) through the Air Force Petroleum Office (AFPET). The authors especially thank Norman Ledgerwood of DSCR, along with Virgil Regoli and Steven Shaeffer of the AFPET for their support of this effort. The authors also thank Steven Zabarnick and Matthew DeWitt of the University of Dayton Research Institute and Chuck Delaney of Encore Logistics Support Systems, Inc. for their technical support and guidance. The authors also thank Robert Rogers of the United States Air Force Academy for his assistance in the laboratory, and Linda Shafer of the University of Dayton Research Institute for the HPLC polars and GC-MS analysis of selected samples. The authors also thank Charles Bleckmann of the Air Force Institute of Technology for his technical advice.

1. Summary

The goal of this study was to find whether significantly reducing the DiEGME concentration in aviation fuel would result in a change in any anti-microbial properties attributed to DiEGME at the current concentration level. This study evaluated DiEGME's microbial activity at aqueous phase concentrations of ~0-30% (~ 0.0-0.05% by volume in the fuel), compared to the ~30-60% aqueous phase concentrations (~ 0.05-0.15% by volume in the fuel) typically found in aircraft fuel storage tanks. Two types of microbes were challenged in this study: lab cultured microorganisms from the American Type Culture Collection (ATCC), and microorganisms recently collected from commercial aircraft fuel tanks in Roswell, NM and Victorville, CA air bases. These two types of microorganisms were chosen for the current study due to their possible differences in behavior to fuel system icing inhibitor (FSII) exposure. Although some differences were observed, it was nevertheless the case for both lab and field microorganisms that DiEGME concentrations of 10% and above in the aqueous phase significantly reduced and/or eliminated microbial growth. Of the six field microorganisms tested, two persisted at 30% DiEGME. Additional testing was performed at 30-60% DiEGME to determine if these two survived even at current levels of DiEGME. It was found that they did survive, but that the overall microbial consortia growth numbers were lower than without the presence of DiEGME. Greater reduction of microbial growth did not necessarily occur with increases of DiEGME concentration beyond 10%.

2. Introduction

Microbial contamination has long been known to be problematic in aviation fuel (1,2, 3, 4,5). Deleterious effects of microbial action can include: plugging of fuel filters, surface pitting, degradation of fuel and/or additives, and aircraft failure. Microbes use fuel hydrocarbons as a source of energy and they use water that frequently accumulates in aviation fuel systems as a source of nutrients. In addition to encouraging microbial growth, water can also be harmful to fuel systems by itself in the form of ice, which can cause fuel system malfunctions (6). As a result of several instances in the 1950's and 1960's (2, 3, 4, 7,8), the U. S. Air Force (USAF) added ethylene glycol monomethyl ether (EGME) to the specification for military jet fuel as a precautionary fuel system icing inhibitor (FSII). The EGME also acted to deter microbial growth in aviation fuel (9, 10). In 1984, the U.S. Navy (USN) added another, less toxic FSII to the JP-5 specification, diethylene glycol monomethyl ether (DiEGME). DiEGME also proved to be an effective deterrent to microbial growth in aviation fuel. In the early 1980s, the USAF replaced EGME with DiEGME due to toxicity and flashpoint concerns surrounding EGME; the Navy adopted DiEGME in its JP-5 primarily due to its higher flashpoint (2, 4, 11, 12). Several studies have explored the effectiveness of DiEGME and other FSII additives in curbing or eliminating microbial growth (13, 14, 15, 16, 17, 18, 19). These studies generally recommended that FSII levels of 15% or greater in the aqueous phase must be maintained for the control and/or elimination of microbial growth.

Years after the introduction of DiEGME into USAF fuel systems, the Air Force has seen increased operational problems due to the effects of DiEGME. DiEGME has been implicated in fuel tank topcoat peeling in the B-52 and in the disarming of filter coalescers. In addition, the high dosage concentration of DiEGME has meant significant cost incurrence for the Air Force. As a result, there is a study being conducted to determine whether the FSII concentration can be lowered to ameliorate these problems, while at the same time retaining FSII's desirable traits, such as the prevention of ice crystal formation in fuel and control of microbial growth (20).Over the same period of time, the Air Force has also seen an increase in incidents related to microbial contamination. It has been hypothesized that the number of microbes resistant to DiEGME has been gradually increasing, resulting in more maintenance issues in fuel systems (21). In addition to the concerns of the larger minimum FSII effort, there is also some contention as to whether DiEGME is still an effective biocide/biostat, and if so, what minimum concentration in fuel and/or aqueous phase is required for it to be effective (17, 19, 21, 22).

The current study was undertaken to consider issues concerning microbial activity of DiEGME at reduced concentrations that were not wholly addressed in earlier, similar studies. No study prior to the current one addressed the biostatic/biocidal activity of DiEGME at low levels on standard lab consortia tested in the past, and also made some attempt to represent the current common consortia most likely to be found in the field. (Further information on these microbes appears in sections 3.2 and 3.3.) Although it is not possible to truly represent either the sheer variety or the relative numbers of microbial contaminants in aircraft fuel, due to many sampling variables such as regional temperature variability, free water differences, humidity, and aircraft tank geometry differences, a large wing tank sampling study conducted by our lab in 2004-2006, ranging over 93 aircraft, 15 airframes, and 14 airbases made it possible for a fairly representative microbial sampling consortia to be available for the current study (21, 23). Five bacteria and one fungus isolated from Roswell and Victorville air bases were chosen to represent wild consortia for this study, based on the high frequency of occurrence of their genera and/or species in the overall sampling study (21). (Further information on the microbe selection process appears in section 3.3.)

The methodology of the current study is modeled after that of the Phillips report of 1964 (24), which used 100 mL French square bottles with 35 mL fuel and 50 mL Bushnell Haas (BH) solution to simulate tank conditions, and traditional plate colony counts to evaluate microbial growth levels. The Phillips methodology was chosen due to the following factors: the materials needed were already at hand, the laboratory space required was small, and the report contained some guidance for interpretation of colony counting results. The Phillips report addressed the anti-microbial activity of EGME at low (0-30% by volume in the water phase) concentrations using an unknown mixed microbial culture from the field.

The report suggested that 15% EGME in the aqueous phase was adequate to control microbial growth. The Phillips report, however, did not include DiEGME.

Another paper, published by Hill in 2005, explores the microbial activity of DiEGME (19). Hill's paper included both field and lab grown (ATCC) microorganisms to evaluate DiEGME's effectiveness at low concentrations. DiEGME concentrations used in the Hill study ranged from 0-19% by volume in the aqueous phase. Hill's results did not show any differences between field and lab microbes in terms of their resistance to DiEGME, but the paper did demonstrate how exposure to small amounts of DiEGME could gradually increase microbial resistance to the additive in field microbes. The Hill paper also suggested that DiEGME levels of 15% by volume and above in the water phase would be expected to be adequate for control of microbial growth, a fraction of the current typical concentration.

The current study takes the methodology provided by the Phillips paper and applies it to some of the issues explored in the Hill experiments with DiEGME. This study utilizes a larger group of microbial samples than Phillips or Hill, and it also illustrates some differences between lab grown and field consortia responses to DiEGME. As a result, the current study provides additional valuable information regarding potential effects of decreasing the concentration of DiEGME in the fuel system, and it also provides insight into the current role of DiEGME with respect to microbial contamination.

Determining an appropriate minimum dosage for DiEGME, the current FSII, is complex. FSII concentration in the fuel and/or aqueous phase varies according to temperature, free water concentration, aircraft system geometry, and flight mission (20). FSII is generally added at three to five times the lowest amount required for prevention of ice crystal formation, as losses in the aircraft system during normal operation are expected. Although commercial aircraft operate safely without FSII, military use typically involves lower fuel turnover, which increases storage times, allowing more water to accumulate and thus improving the likelihood of ice crystal formation and microbial contamination. As a result, military aircraft may generally have a greater need for FSII than commercial aircraft. Previous work suggests that 0.04% DiEGME in the fuel phase (~25% in the aqueous phase) is adequate for ice crystal suppression (20). This study's purpose was to find whether this level of DiEGME is also adequate for microbial growth suppression. Furthermore, this study attempted to determine what the minimum level of DiEGME in the aqueous phase is necessary for keeping microbial growth in check.

Because DiEGME levels are frequently expressed in terms of the concentration required in the aqueous phase in this paper, it is useful to see how the level in the aqueous phase relates to the actual amount of DiEGME added to the fuel. The chart below by DeWitt et. al shows the relationship between the amount of DiEGME added to the fuel phase and the volume of DiEGME expected in the aqueous phase (20).

This chart was used in the current study to link DiEGME fuel additive dosage amounts with corresponding additive amounts in the aqueous phase.

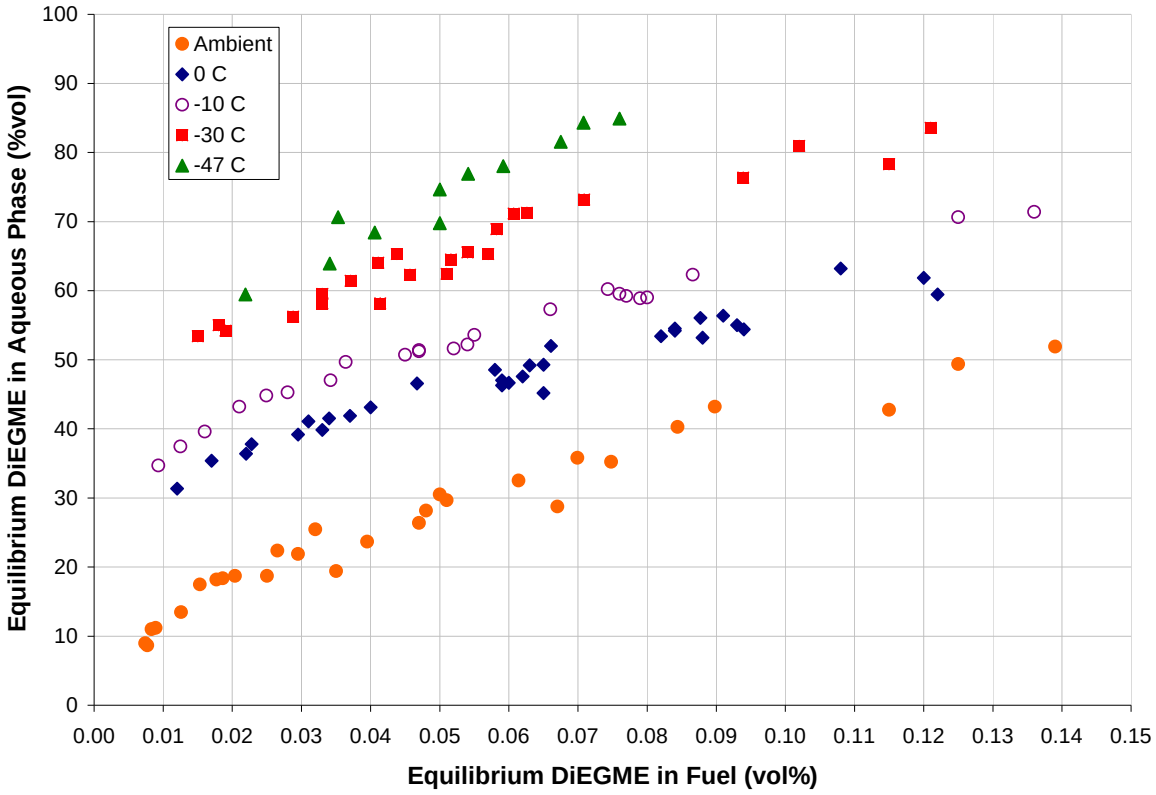

Figure 1. Equilibrium DiEGME concentration in fuel vs. aqueous phase

3. Methods, Assumptions, and Procedures

3.1 Materials

Clear French square 100 mL bottles from Fisher Scientific were sterilized by autoclave. The test setup for each French square consisted of 35 mL of Jet A aviation fuel POSF 4877 for the fuel phase and 50 mL of water phase made from full-strength Bushnell-Haas broth nutrient solution (Sigma-Aldrich, Inc. St. Louis, MO) with DiEGME added as appropriate. Fuel was filtered with a 0.45µm hydrophobic cellulose nitrate filter (Nalge Nunc, Rochester, NY) prior to use in the test setup. The Bushnell-Haas solution was sterilized by autoclave. Microbes were cultured on Luria-Bertani (LB) agar plates. LB broth and Difco granulated agar were obtained from Sigma-Aldrich and Becton-Dickinson (Sparks, MD), respectively. All plating was performed in a laminar flow hood. A Reichert Quebec Darkfield colony counter (Depew, NY) was used to quantify microbial growth.

3.2 Test Microorganisms

Lab culture microorganisms were obtained from ATCC. They included: *Pseudomonas aeruginosa* (ATCC catalog # 33988), *Hormoconis (Cladosporium) resinae* (ATCC # 20495), and *Yarrowia (Candida) tropicalis* (ATCC # 20336). *P. aeruginosa* is a type of bacteria, *C. resinae* is a fungus, and *C. tropicalis* is a yeast. The *P. aeruginosa* originated from a fuel storage tank in Ponca City, OK, and was deposited into the ATCC collection by R. Allred in 1982. The *C. resinae* originated from an aircraft fuel tank and was deposited by J. J. Marshall from the NLABS collection in 1977. The *C. tropicalis* was deposited in 1971. The ATCC microorganisms were chosen based on their prevalence in the literature; for example, they are used in the ASTM method E 1259-01 for evaluating antimicrobials in liquid fuel (25), and they are used by Neihof, Westbrook, and Hill (13, 16, 19). Microorganisms obtained from Roswell and Victorville aircraft fuel tanks in 2004-2005 included: a *Methylobacterium* species, a *Pseudomonas* species, *Bacillus licheniformis*, *Clostridium intestinale*, *Rhodococcus equi*, and *Hormoconis (Cladosporium) resinae*. All are bacteria except for *C. resinae*. These field microorganisms were the most common found overall in the most recent study of microbial contaminants in aircraft fuel, and were chosen to roughly approximate a realistic test set (21). Due to the fact that microbial consortia are typically not dispersed in fuel systems in a homogeneous manner, it may not be possible to truly capture a representative picture of microbial growth over an entire fleet of aircraft (25). The extensive sampling efforts undertaken by this lab in 2004-2005 were, however, a good recent attempt at representing the likely widespread classes of consortia encountered in the field today (21).

3.3 Wild Type Microbe Collection

The wing tanks of several civilian aircraft in long term storage were sampled from 2004 to 2005. Military aircraft were also sampled throughout 2005. Preliminary results were compiled in a previously published technical report (21). The Victorville and Roswell microbes were obtained from wing tanks on various commercial aircraft that had been idle for at least a year. All Victorville and Roswell aircraft fuel tank samples contained Jet A fuel with water bottoms. DiEGME levels were not recorded for the commercial aircraft during the 2004-2005 sampling study. It is not known whether the Victorville and Roswell commercial airbase aircraft systems, fuel, or fuel microbes were ever exposed to DiEGME. The military aircraft in the 2004-2005 study, however, were known to be exposed to DiEGME in many cases, and in many instances data on DiEGME levels was recorded (23). The frequency of different types of microbial contaminants from 2004 to 2005 in both commercial and military aircraft was noted in the 2004-2005 sampling study, with the six most common overall being chosen to represent the field consortia in the current study. The microorganisms cultured from the field were identified by 16S ribosomal DNA sequencing as: *Pseudomonas* sp. (obtained from a DC-8 aircraft wing tank, Roswell, NM

air base), *Bacillus licheniformis* (727-200 aircraft wing tank, Roswell), *Clostridium intestinale* (DC-8 wing tank, Roswell), *Rhodococcus equi* (767-200 wing tank, Victorville, CA air base), and *Methylobacterium* sp. (767-200 wing tank, Victorville). Field microbes from the commercial planes were chosen to represent typical consortia for both military and commercial aircraft simply because the commercial aircraft yielded far more culturable specimens. Representatives of all the major microbial consortia groups found—that is, the most commonly represented in DNA identification sequences from both the commercial and military aircraft—were only successfully cultured from the commercial aircraft.

Sequencing was performed by MWG Biotech of High Point, NC. Fuel sampling procedures, DNA extraction, purification, and sequencing procedures are detailed elsewhere (11, 21, 23). Procedures used for bacterial sequence identification are also listed elsewhere (11). *Cladosporium resinae* (Roswell) was identified by light microscopy, performed by Forensic Analytical of Rancho Dominguez, CA.

3.4 Test Procedure

All microorganisms were revived from frozen cultures stored at -80º C. They were incubated in 5 mL of LB broth, POSF 4877 fuel + BH broth, and BH broth alone. When visible inspection showed significant microbial growth (indicated by cloudiness or an increase of solid or fluffy material at test tube bottom) of the LB, the fuel + BH, and/or the BH test tubes, the cultures were deemed viable. Two hundred microliter portions of each microorganism's BH test tube were then pipette separately in the case of the single organism tests, or they were pipetted and combined to make a mixed culture in the case of the mixed culture tests. One hundred microliters of the single or mixed culture were then used to inoculate each French square bottle at each DiEGME test level. French square bottles were incubated at 28º C. At the time of initial plating, referred to as Day 0, the microbes were exposed to DiEGME for at least 4, but no more than 24 hours. Colony counts were not taken prior to the Day 0 plating. For all test points, the fuel/water French square set ups were manually shaken for 30 seconds, the phases were allowed to re-separate, and a 100 µL aliquot was drawn from the aqueous phase. The aliquot was spread on an LB plate. A second aliquot was used to make dilutions as needed with the BH, typically 1:100, 1:1000, and/or 1:10,000. Growth rates were microorganism dependent, with colonies typically appearing 24 to 72 hours after plating.

Following this period, the colony plate count was taken by touching each colony with the colony counter probe. The countable range for a raw plate is between 30 and 300 colony forming units (CFU)(26). In practice, however, colonies were sometimes above or below the countable range, despite the dilutions performed. Due to the dilution method used, the maximum corrected raw colony count in the present experiments was 30,000,000 per mL; numbers above 30,000,000 per mL were considered to be too numerous to count (TNTC). This corresponded to a raw count of 300 or above on a plate with a 1/10,000 dilution. Colony counting error is expected to be plus or minus an order of magnitude. This error is based on colony counting results obtained from random, multiple platings. Most of the colony counts reported here were the results of single platings, though random multiple platings were performed throughout the test. Although there is not always a direct relationship between colony count and microbial contamination of aviation fuel—due to the fact that many microbes in aviation fuel and in the general environment may not be culturable on agar plates (27)—it is safe to assume that relationship in the current study, as all of the microbes utilized have been previously cultured on agar plates. In addition, it is often the case that a large colony count is directly suggestive of a significant potential for microbially-induced problems. However, no numerical standards have been universally accepted which define a particular colony count level as problematic (28).

The rest of the essential test procedure used in this study is based on a Phillips report from 1964(24). Essentially, the Phillips method requires plating of the liquid test setups approximately every three days during a 46 day test duration. Control fuel/water mixtures were also maintained throughout the test cycle for each DiEGME concentration level. DiEGME concentration levels tested in this study were: 0, 5, 10, 15, 20, and 30% by volume in the water phase. Controls (fuel/water mixtures with no inoculants added) at each level were also plated randomly throughout the test period. These showed no growth,

suggesting sterile technique. The total fuel phase for each setup was 35 mL, and the total DiEGME/Bushnell-Haas water phase for each setup was 50 mL. An additional study was performed for the field microbes at 30, 40, 50, and 60% by volume in the water phase, due to the fact that some of the field microbes were able to survive at the upper threshold of the original study, which was 30%.

As a side note, experiments were also performed to determine if it was possible to detect cumulative microbial activity following the 46 day test period via an already established HPLC polars analysis and standard GC-MS (29). The polars analysis results are shown in the Appendix.

4. Results and Discussion

4.1 ATCC Microorganism Tests

In these tests, *Pseudomonas aeruginosa*, *Cladosporium resinae*, and *Candida tropicalis* obtained from ATCC were revived from separate frozen cultures. They were tested singly and collectively for their resistance to DiEGME at low additive concentration levels. These microorganisms were grown in the test setups, plated on LB plates, and their colonies were counted after 72 hours of incubation for each test point, as *C. resinae* colonies were not clearly visible prior to 72 hours. Three types of information are shown below: 1) Figures 2-8 show French square test setups following the 46 day test duration, which present visual comparisons of the liquid ATCC inoculated samples at different DiEGME concentrations; 2) Figures 9-16 show agar plate growth of the ATCC consortia, ATCC *Pseudomonas* alone, ATCC *Cladosporium (Hormoconis)* alone, and ATCC *Candida* alone, at several different points during the experiment. The agar plates shown were used for visual inspection and/or enumeration of colony growth; 3) Figures 17-20 below summarize ATCC microbial growth for the 46 day test period for the mixed ATCC consortia and also for each of the ATCC microorganisms singly.

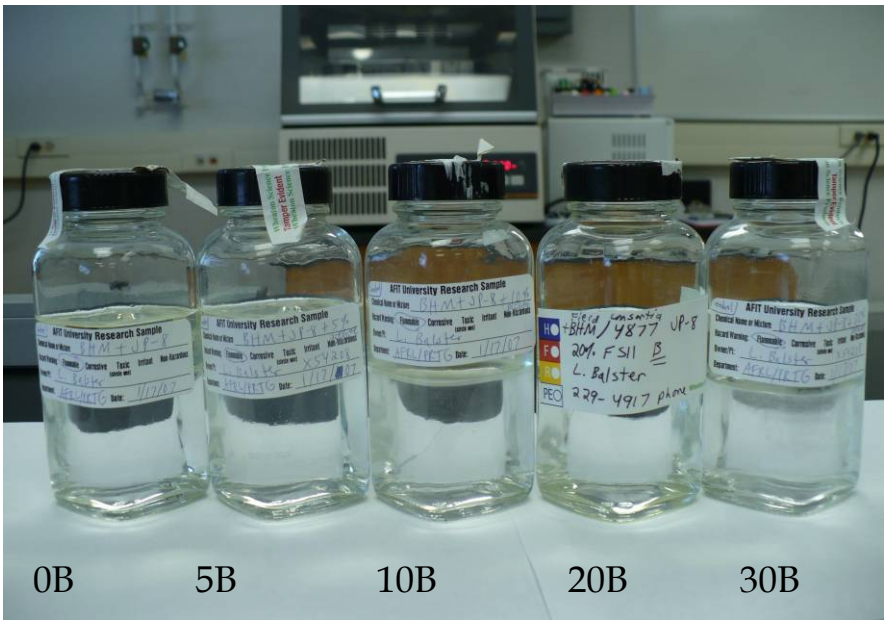

Figure 2. Fuel/Bushnell Haas French square blanks (denoted by a B) following 46 day testing. From left to right, DiEGME concentrations are: 0, 5, 10, 20, and 30% by volume in water phase.

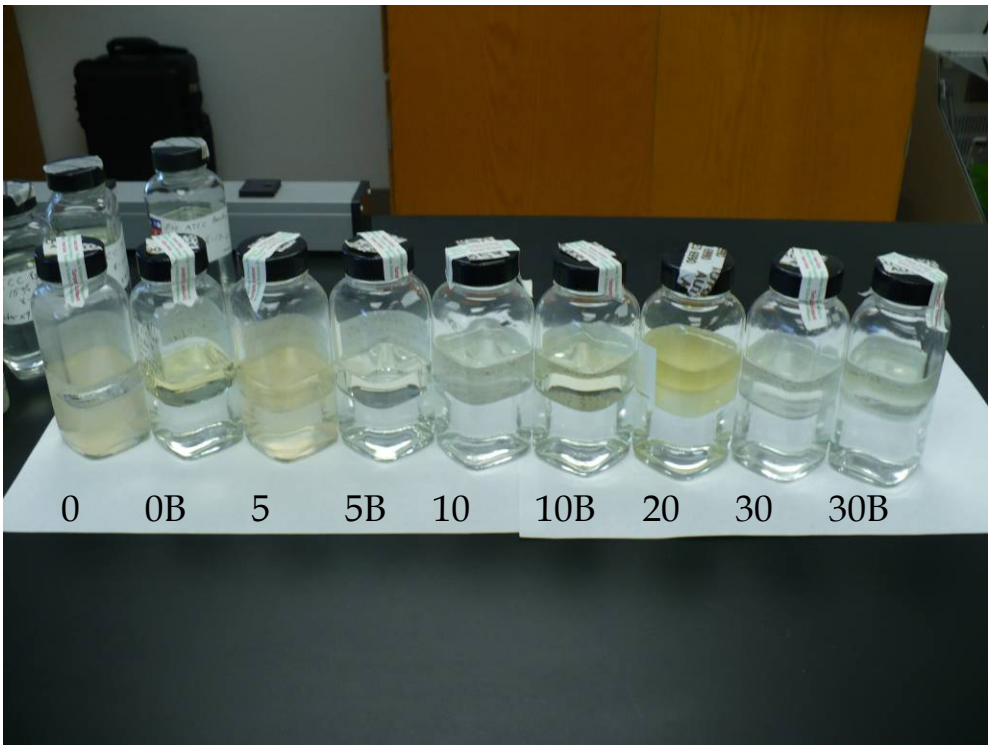

Figure 3. ATCC consortia following 46 day test, side by side with respective blank, except for 20%. DiEGME concentrations are, from left to right: 0, 5, 10, 15, 20, and 30% by volume in aqueous phase

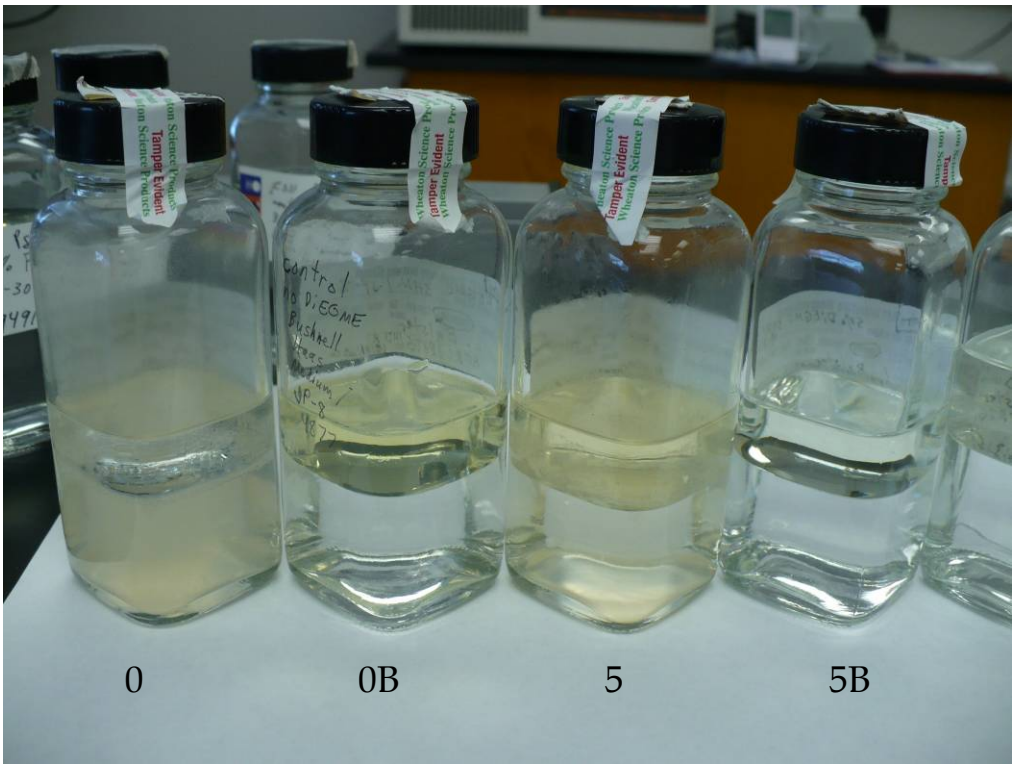

Figure 4. ATCC consortia following 46 day test, side by side with respective blank. DiEGME concentrations are: 0 and 5% by volume in aqueous phase

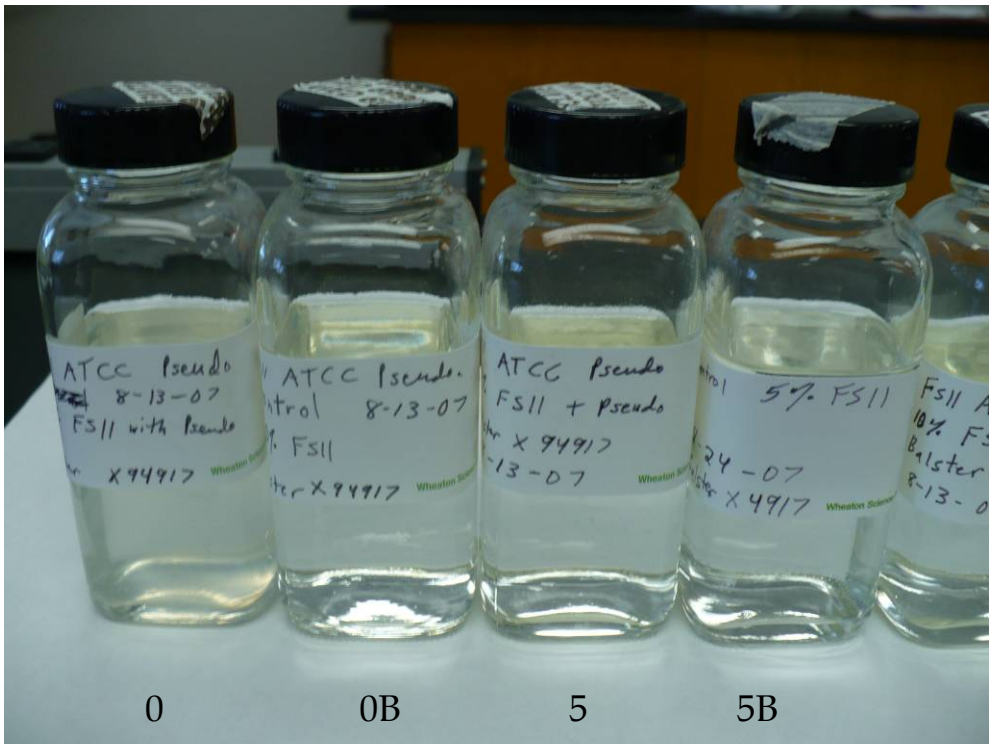

Figure 5. ATCC *Pseudomonas* following 46 day test. DiEGME concentrations are: 0 and 5% by volume in aqueous phase, paired with respective blanks

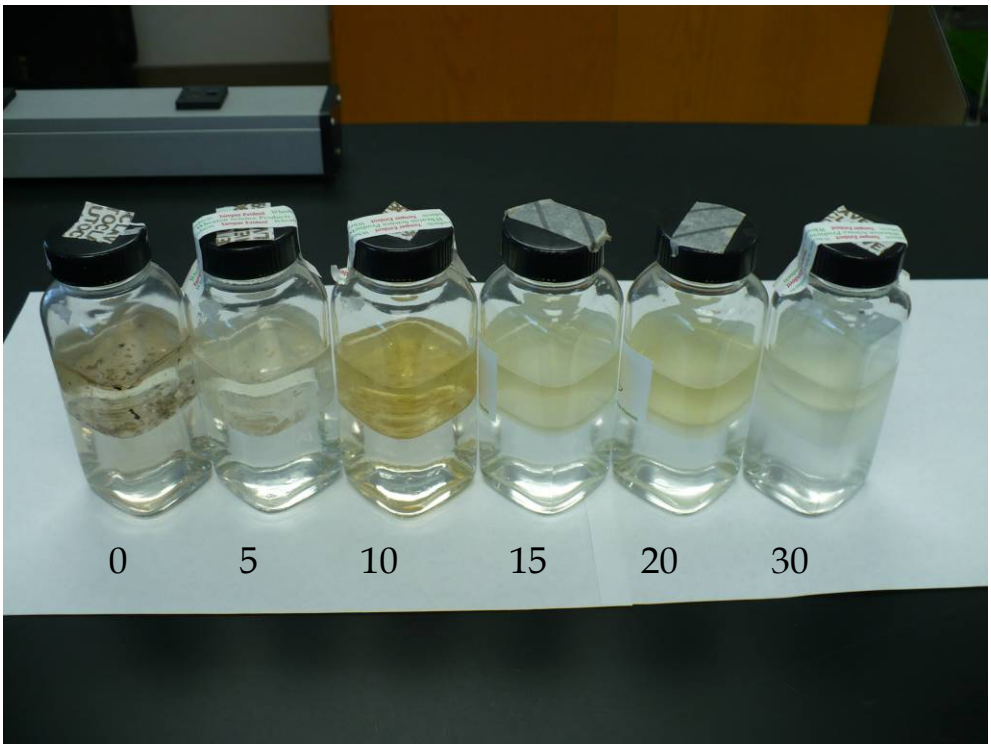

Figure 6. ATCC *Cladosporium* following 46 day test. DiEGME concentrations are: 0, 5, 10, 15, 20, and 30% by volume in aqueous phase

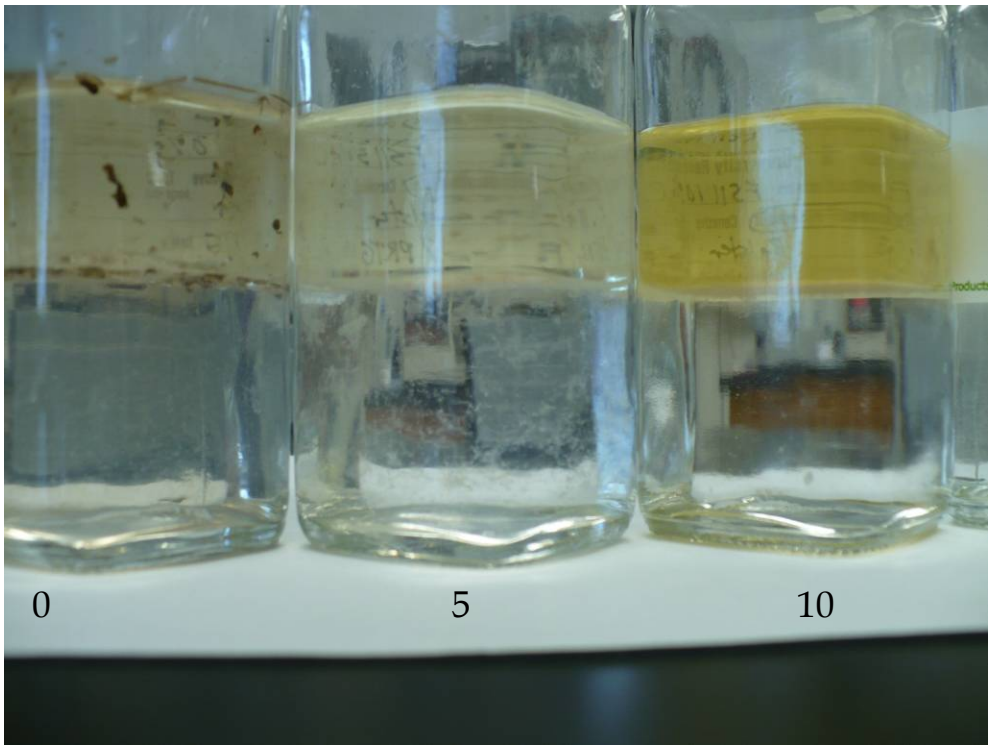

Figure 7. ATCC *Cladosporium* following 46 day test. DiEGME concentrations are: 0, 5, and 10% by volume in aqueous phase

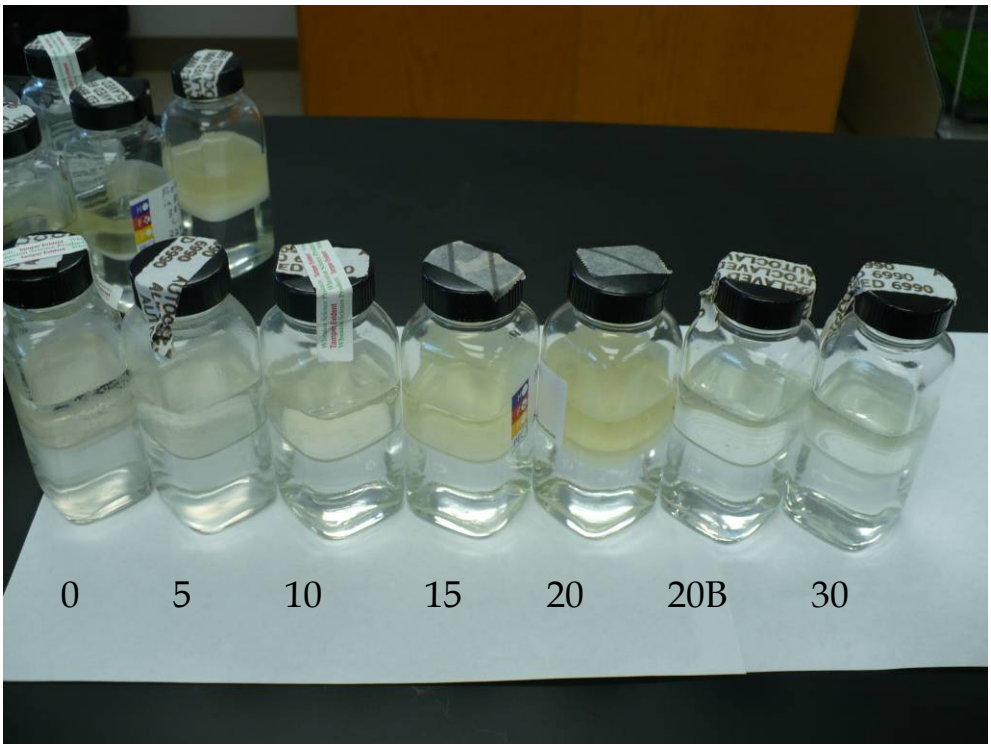

Figure 8. ATCC *Candida* following day 46 of test. DiEGME concentrations are: 0, 5, 10, 15, 20, and 30% by volume in aqueous phase, with 20% next to 20% blank.

Several observations become apparent from visual inspection of the ATCC consortia test setups, as well as those from the separate ATCC microorganisms. The blanks at all FSII levels, shown in Figure 1, are clear in all cases, as would be expected, as clarity of the water phase suggests lack of growth. In the inoculated 0% DiEGME test setups, shown in Figures 2-8, cloudiness in the water phase is apparent for all ATCC setups. Cloudiness that doesn't dissipate is considered to be a very good indicator of microbial activity. In addition, there is particulate buildup in the 0% FSII ATCC consortia, Figures 3 and 4, as well as the 0% FSII ATCC *Candida* (white particles) in Figure 8. In the 0% FSII ATCC *Cladosporium*, shown in Figures 6 and 7, there are especially obvious brown fungal patches present in the fuel phase, as well as white or light brown colored particles in the water phase.

Although it may not be clear from the images above, biofilms were also present in all 0% test setups. In fuel systems, a biofilm is a microbial growth formation that typically appears as a sheen, pellicule, or mat that forms between the fuel and water layers or on the interior sides of a tank. Biofilms consist of microbes, inert detritus, water, and extracellular polymeric substances (EPS)—also known as the glycocalyx, which is a polysaccharide or peptide slime. Biofilms protect bacteria, fungus, and/or yeast and encourage their growth, which in turn promotes the deleterious effects of microbial contamination, such as microbially induced corrosion (MIC) and fuel degradation. The presence of biofilms can also lead directly to the plugging of fuel lines and filters (25).

For the 5% FSII concentration in the aqueous phase, there is still substantial cloudiness in the water phase for the ATCC consortia, shown in Figures 3 and 4, suggesting the presence of significant microbial growth. For the ATCC *Pseudomonas*, however, shown in Figure 5, the 5% FSII level is clear, suggesting considerably less if not zero growth. For the 5 and 10% *Cladosporium*, Figures 6 and 7, there are obvious white particulates in the water phase, as well as a color change in the fuel phase. However, there are substantially fewer particulates in the 10% as compared with the 0 and 5%. The 5% *Candida* in Figure 8 also shows some cloudiness. Unlike the 10% *Cladosporium*, the 10% ATCC consortia, ATCC *Pseudomonas* and ATCC *Candida* showed no cloudiness or particulates at 10%, suggesting much less or zero growth at the 10% concentration. None of the 15-30% FSII levels showed cloudiness in the water phase, or particulates. Although 15 and 20% do have color changes in the fuel phase, it must be noted that the 15 and 20% experiments were added to the biological minimum FSII program after testing was already underway, and the 15 and 20% test points were therefore not performed at the same time as the others. The fuel was also stored in a different glass container before it was used in experimental setups. It seems possible that those color differences were actually due to the aging of the fuel in storage, perhaps related to the formation of hydroperoxides, phenols, or other oxygenates that cause changes in fuel color. In addition, it can be noted that each test setup has a slightly different fuel phase color. Generally speaking, a change in the fuel phase color does *not* necessarily indicate microbial activity.

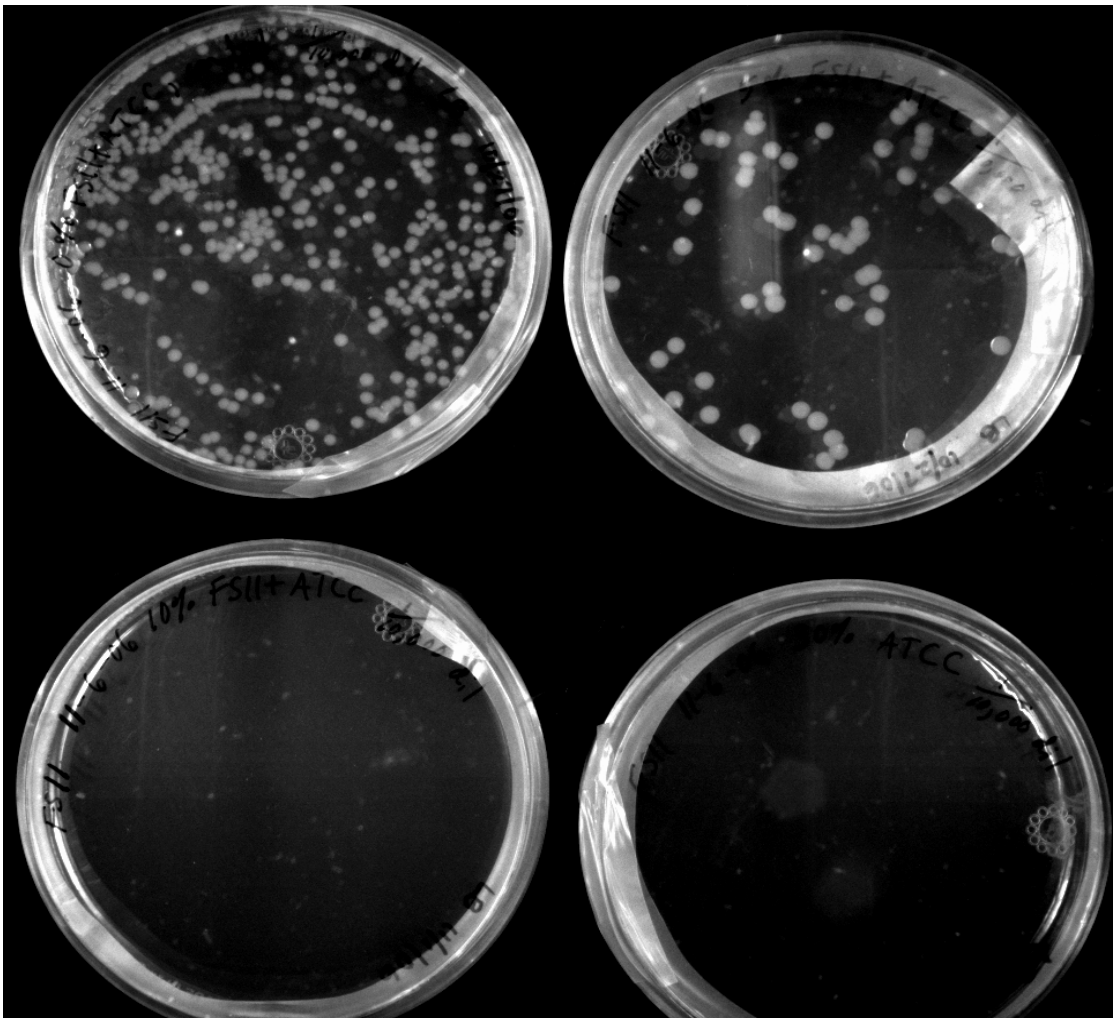

Figure 9. ATCC consortia at day 6 of incubation. DiEGME concentrations, 1/10,000 dilution, are: 0% (upper left), 5% (upper right), 10% (lower left), and 30% (lower right) by volume in water phase. Colonies are only growing at the 0 and 5% levels.

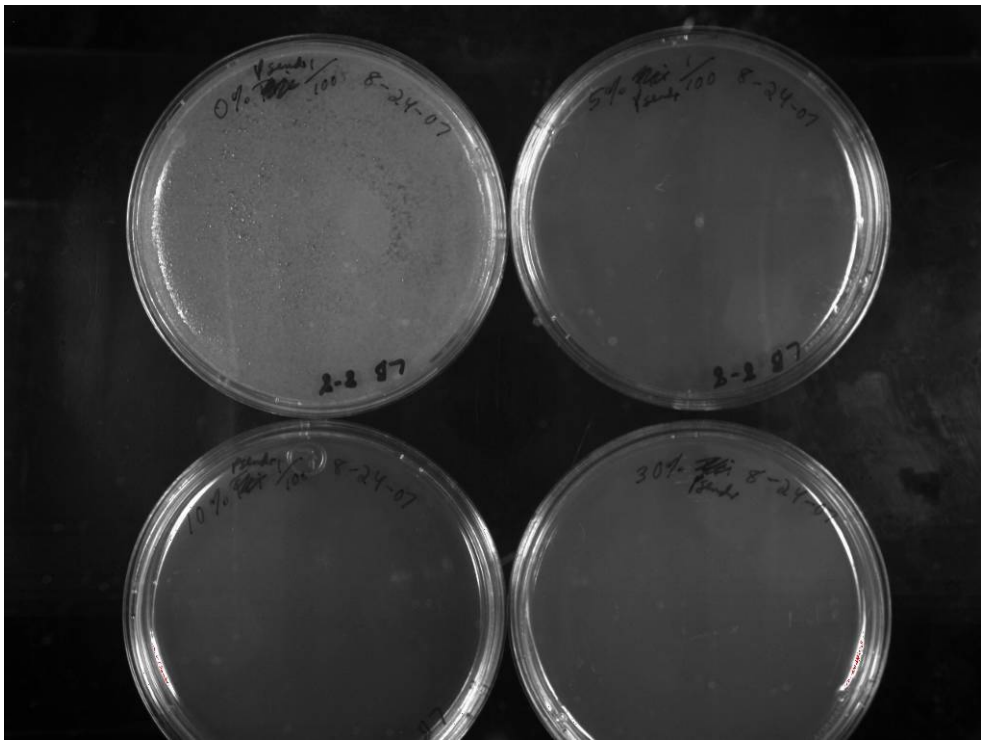

Figure 10. ATCC *Pseudomonas* at day 11. DiEGME concentrations, 1/100 dilution, are: 0,5,10, and 30%. The 0% plate (upper left) is swarming with growth; the other plates have no colonies.

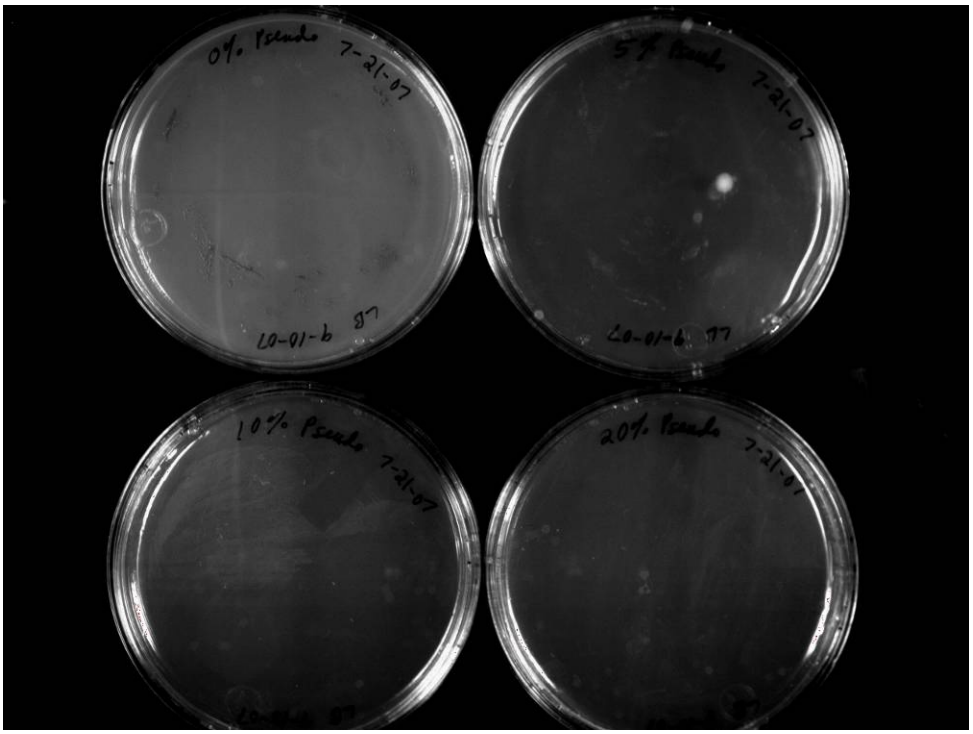

Figure 11. ATCC *Pseudomonas* at day 38. DiEGME concentrations are: 0,5,10, and 20%. The 0% plate (upper left) is swarming with growth; the 5% plate (upper right) has one colony. The 10 and 20% plates (lower left and right) have none.

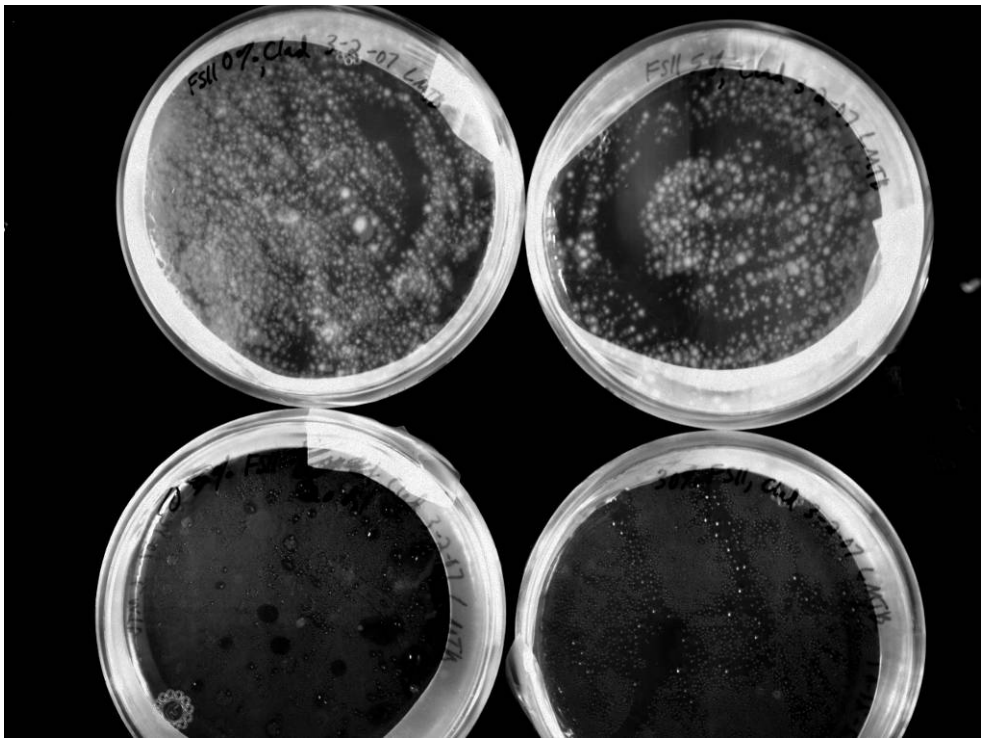

Figure 12. ATCC *Cladosporium* at day 31 of incubation. DiEGME concentrations are: 0 (upper left), 5 (upper right), 20 (lower left) and 30% (lower right). Lower plates have condensation, not colonies.

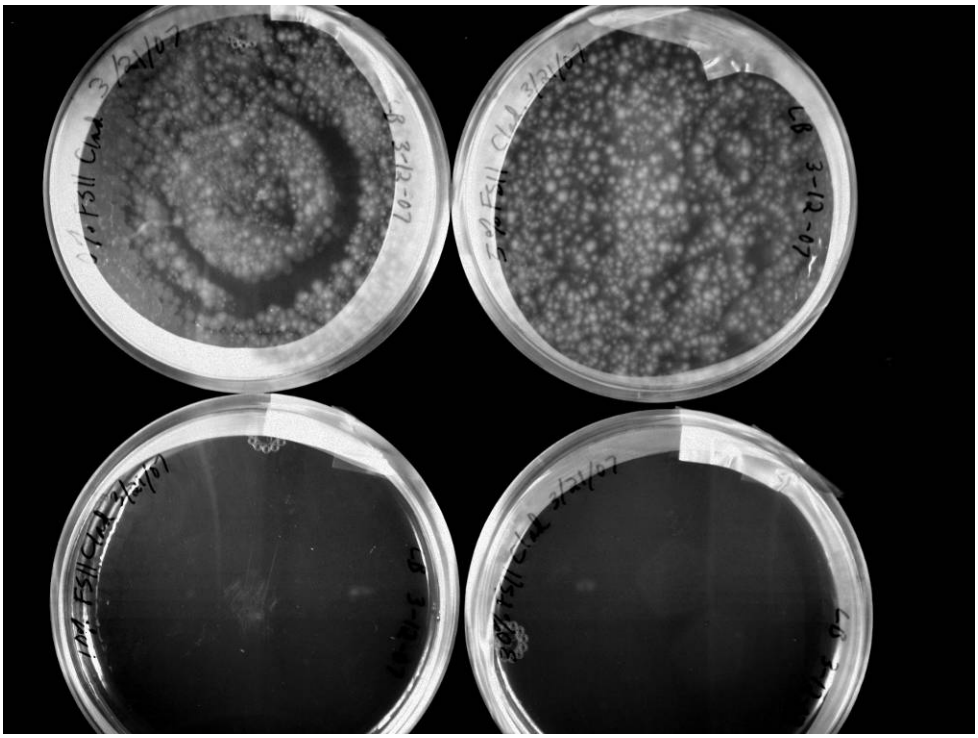

Figure 13. ATCC *Cladosporium* at day 47. DiEGME concentrations are: 0 (upper left), 5 (upper right), 10 (lower left), 30% (lower right). The 10 and 30% plates have no colonies.

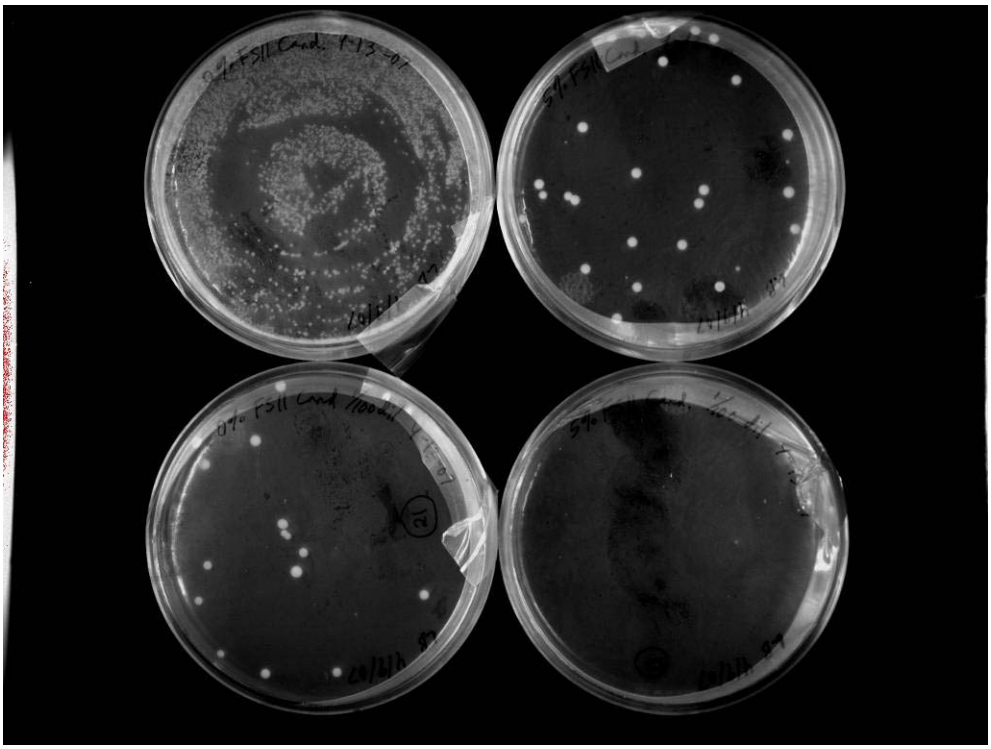

Figure 14. ATCC *Candida* at day 8. DiEGME concentrations are: 0 (no dilution, upper left), 5 (no dilution, upper right), 0 (1/100 dilution, lower left), and 5 (1/100 dilution, lower right). The 5% 1/100 dilution plate has no colonies.

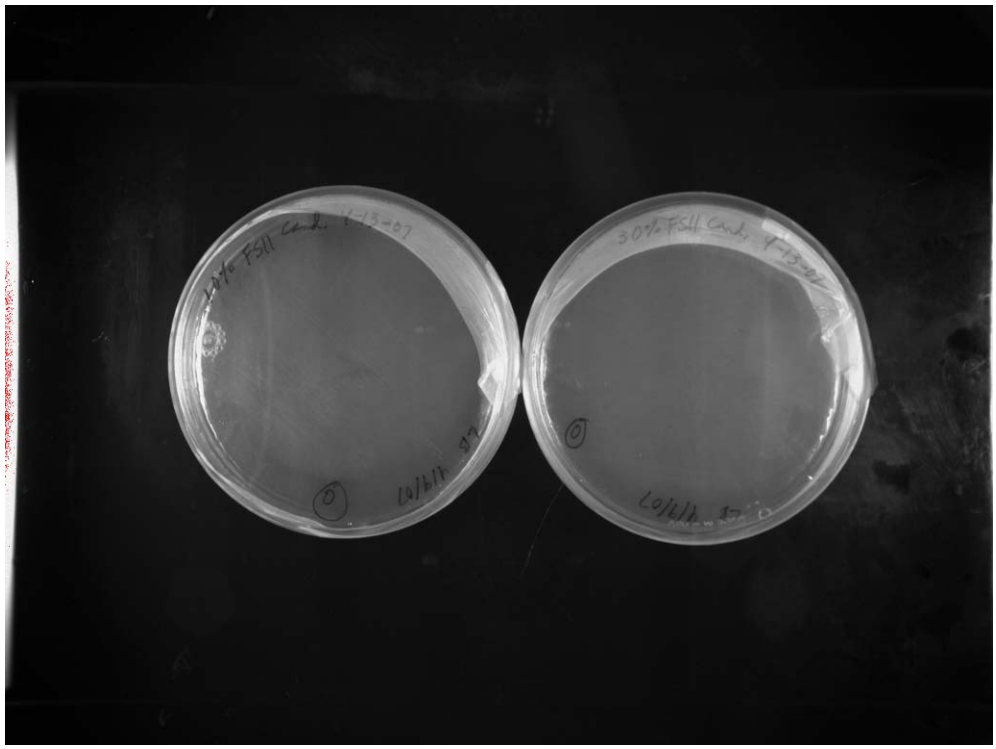

Figure 15. ATCC *Candida* at day 8. DiEGME concentrations are: 10 (left) and 30% (right). Neither plate has colonies.

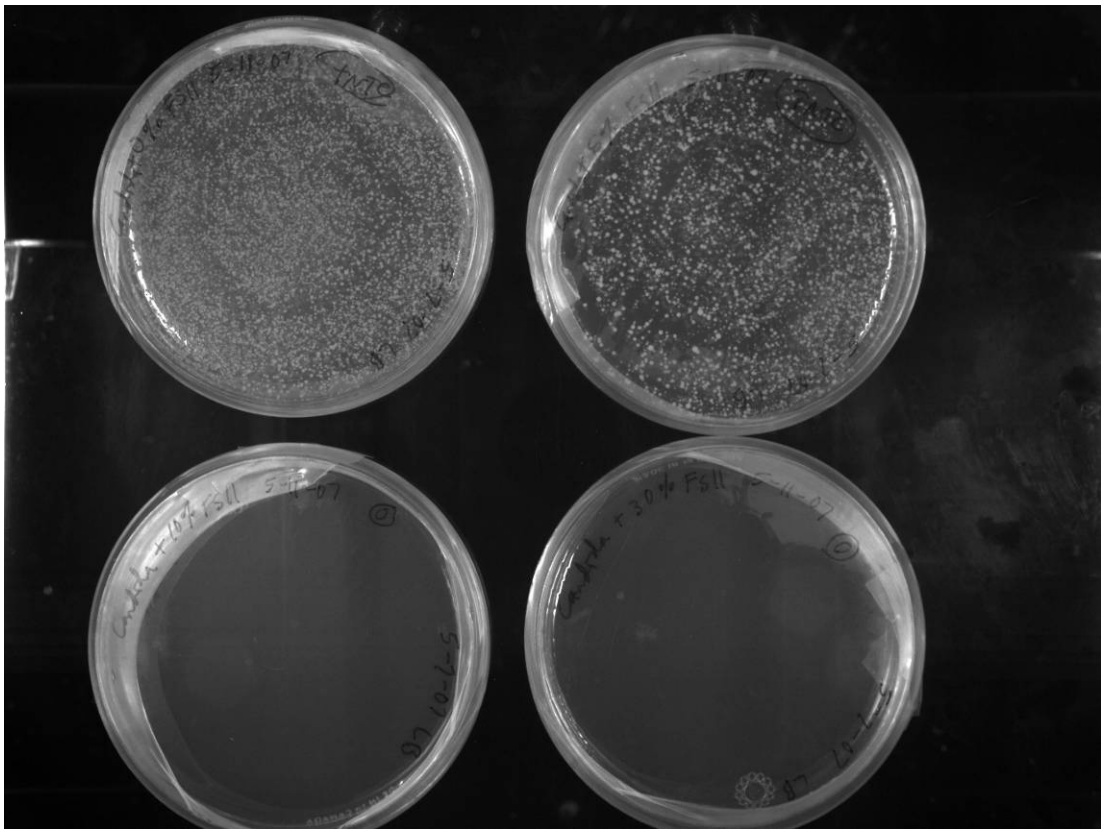

Figure 16. ATCC *Candida* at day 35. DiEGME concentrations are: 0 (upper left), 5 (upper right), 10 (lower left), and 30% (lower right). The 10 and 30% plates have no colonies.

Similar observations can be made concerning the colony growth on the LB agar plates over the 46 day test period. The ATCC consortia plates shown in Figure 9 clearly indicate that 0 and 5% FSII levels permit microbial growth, although 5% has less growth than 0% FSII. The 10% FSII and above levels seem to have the capacity to completely eliminate growth. This is also true for the separately tested microorganisms, including the ATCC *Pseudomonas* plates shown in Figures 10 and 11, the ATCC *Cladosporium* shown in Figures 12 and 13, and the ATCC *Candida* shown in Figures 14, 15 and 16. It is also apparent that the ATCC *Cladosporium* and *Candida* have more resistance to the low 5% level of FSII than does the ATCC *Pseudomonas*.

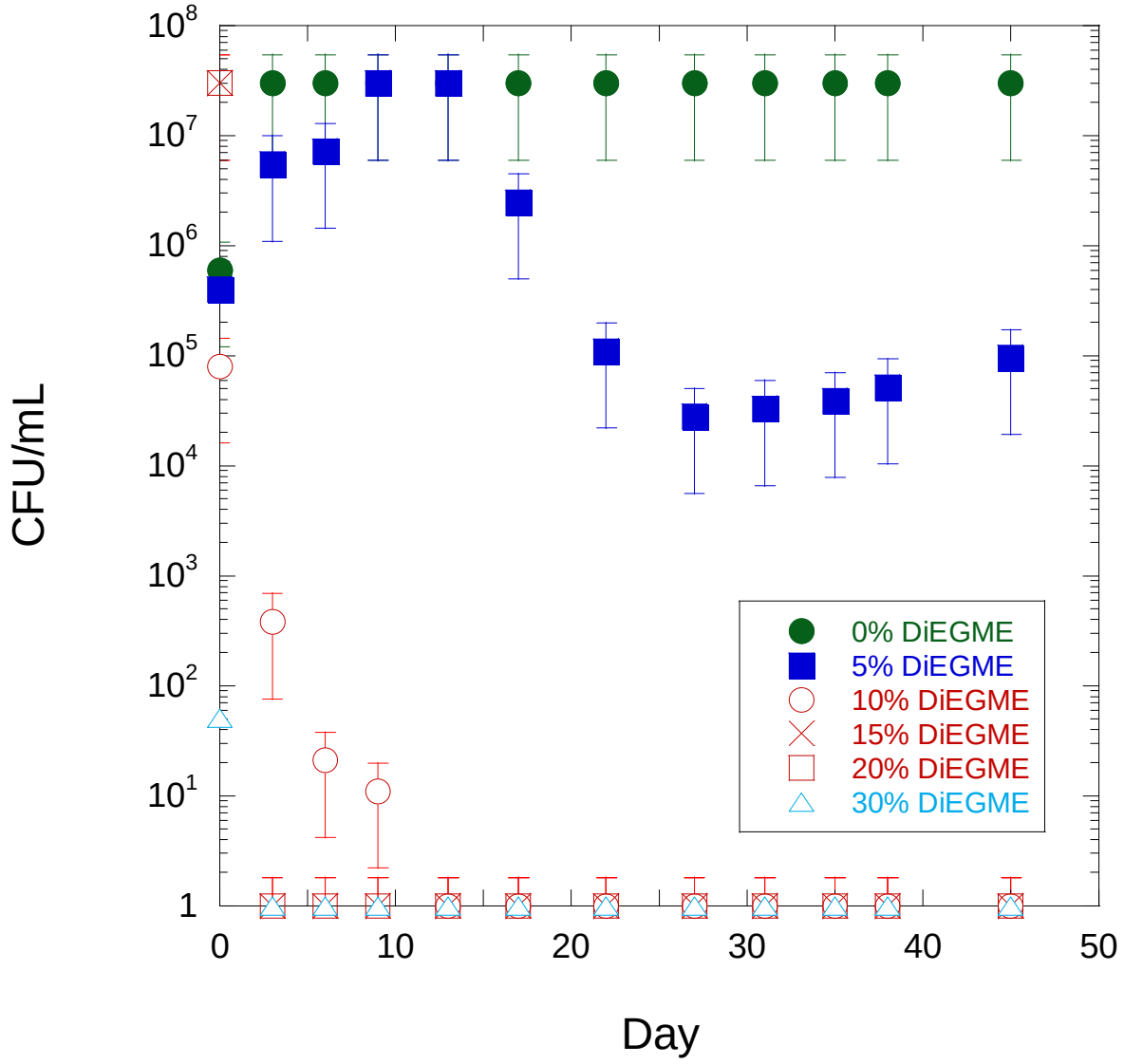

Figure 17. Semi-log plot of ATCC consortia colony forming units (CFU) per mL of liquid sample over the 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

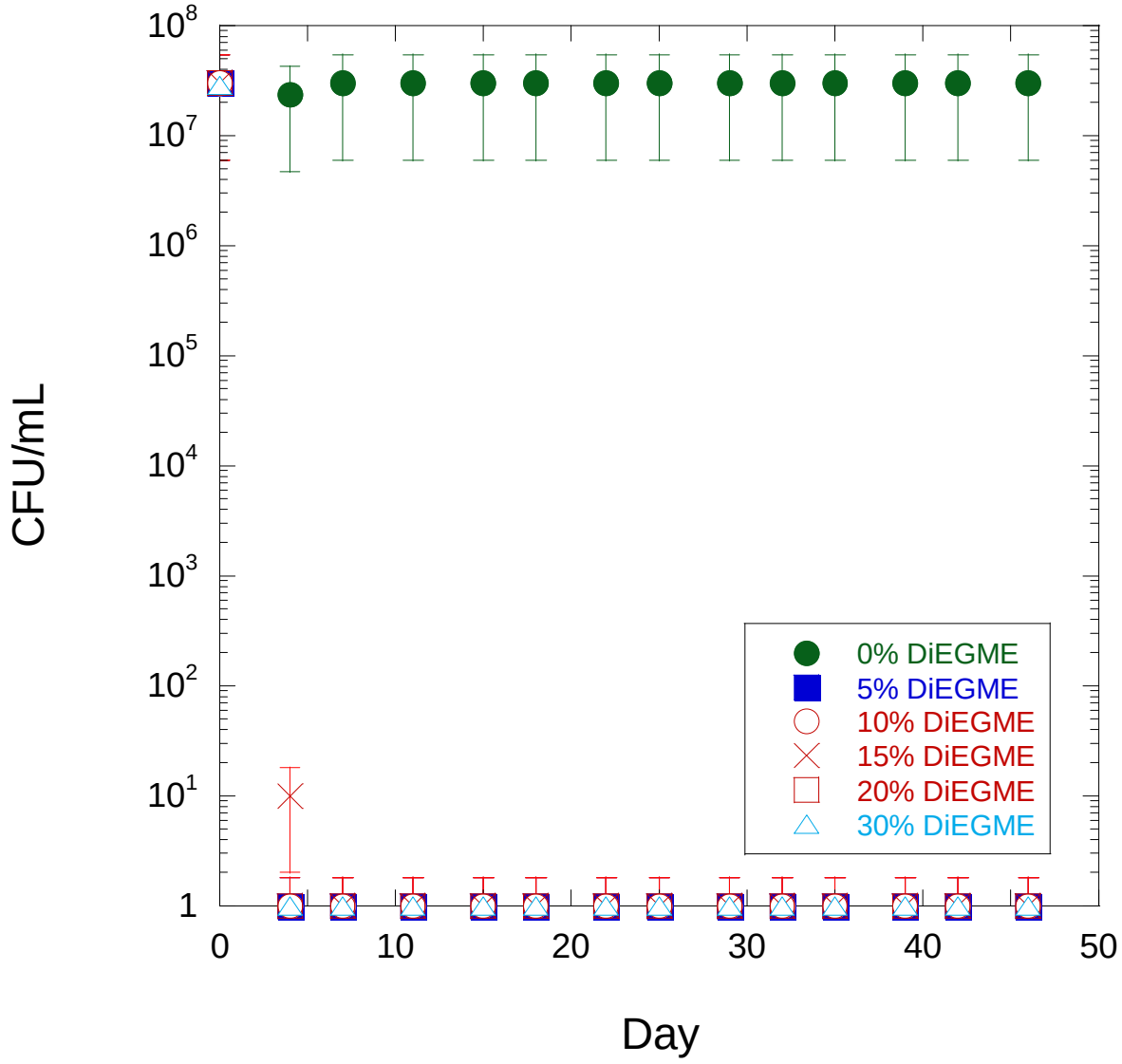

Figure 18. Semi-log plot of ATCC *Pseudomonas* colony counts over a 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

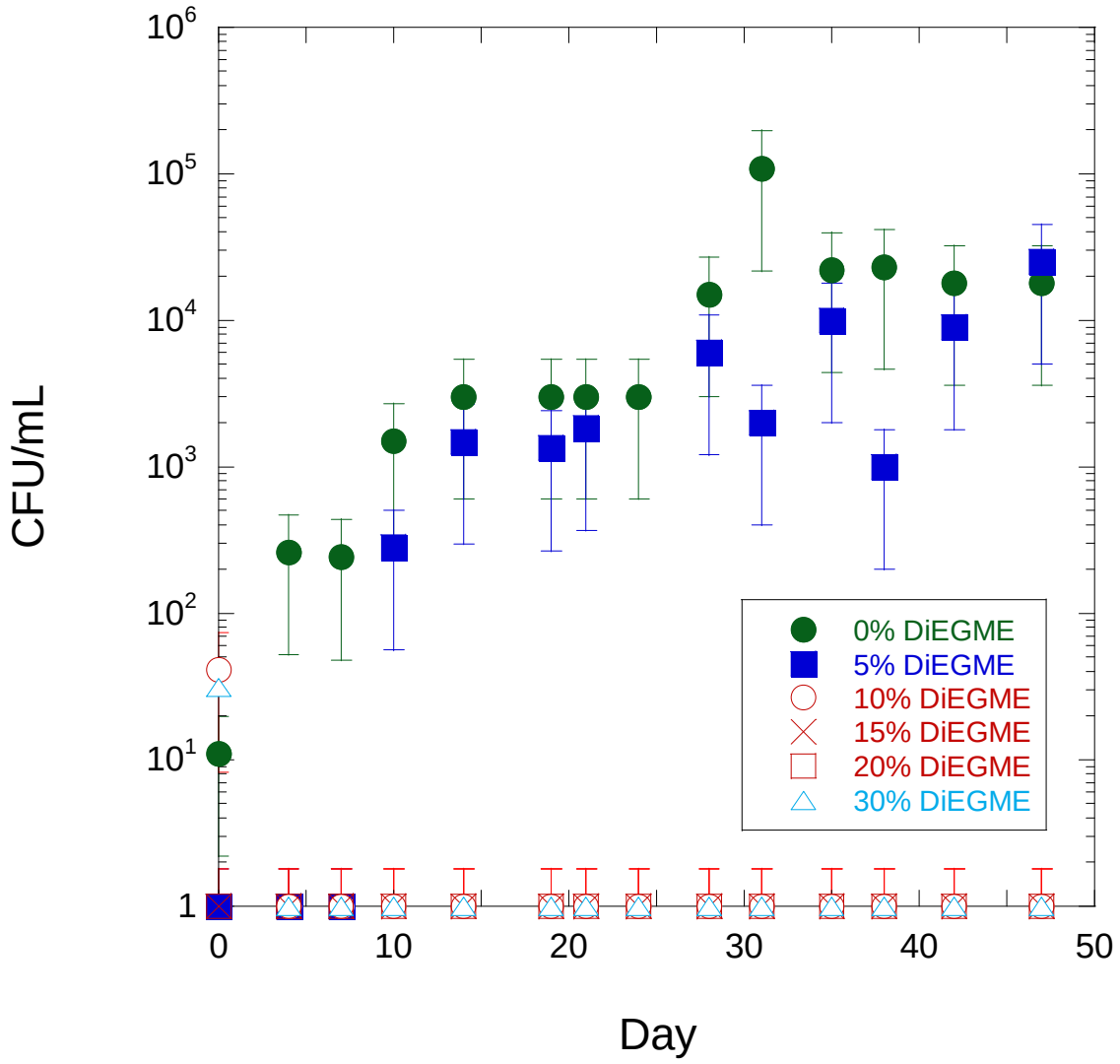

Figure 19. Semi-log plot of ATCC *Cladosporium* colony counts over a 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

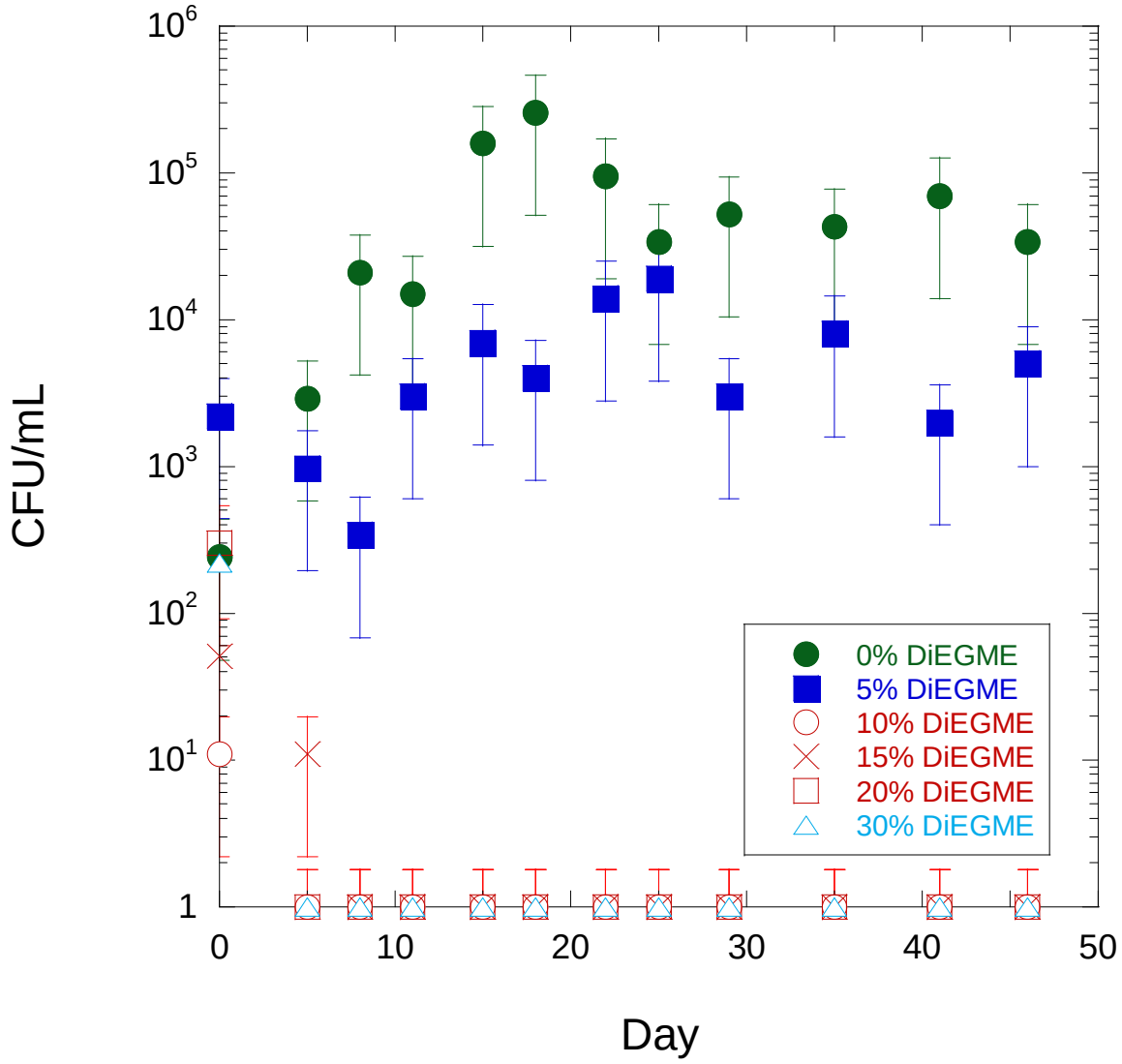

Figure 20. Semi-log plot of ATCC *Candida* colony counts over a 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

It is clear from Figures 17-20 that *Pseudomonas* produces the greatest number of colonies compared to the other ATCC microorganisms, but it also experiences the greatest inhibition due to DiEGME, with only 5% DiEGME completely eliminating its population after 4 days. Although the *Candida* and *Cladosporium* produce fewer colonies, their growth continues unabated at the 5% level, though it is also halted at 10% DiEGME in nine days or fewer. The ATCC consortia test containing all three microorganisms shows similar results, though the growth patterns of the consortia are somewhat different, most likely due to interaction among the three types of microorganisms and their metabolites. The microorganisms at all levels of DiEGME treatment still showed growth after four hours exposure to the icing inhibitor. However, as early as Day 1, there were significant declines in colony count for all DiEGME levels above 5% by volume in the water phase. Results suggest that a DiEGME level of 10% by volume in the water phase is adequate for elimination of microbial growth for these microorganisms. This level is slightly lower than that suggested by previous studies.

4.2 Field Microorganisms Consortia Test

Six microorganisms were obtained from the field, cultured, then frozen at -80º C. These microorganisms included: *Pseudomonas* sp., *Rhodococcus equi, Bacillus licheniformis, Clostridium intestinale, Methylobacterium* sp., and *Cladosporium resinae*. They were revived from the frozen state separately by incubation at 28ºC on LB agar plates, then utilized in the same procedure listed above. The field microorganisms were tested as a consortia only, due to time constraints of the study. The same DiEGME levels were used as above. Figures 21 and 22 below show the French square test setups following the 46 day test. Figures 23-25 below show agar plate growth at several different points during the experiment. Figure 26 shows colony counts at all DiEGME levels over the 46 day test period.

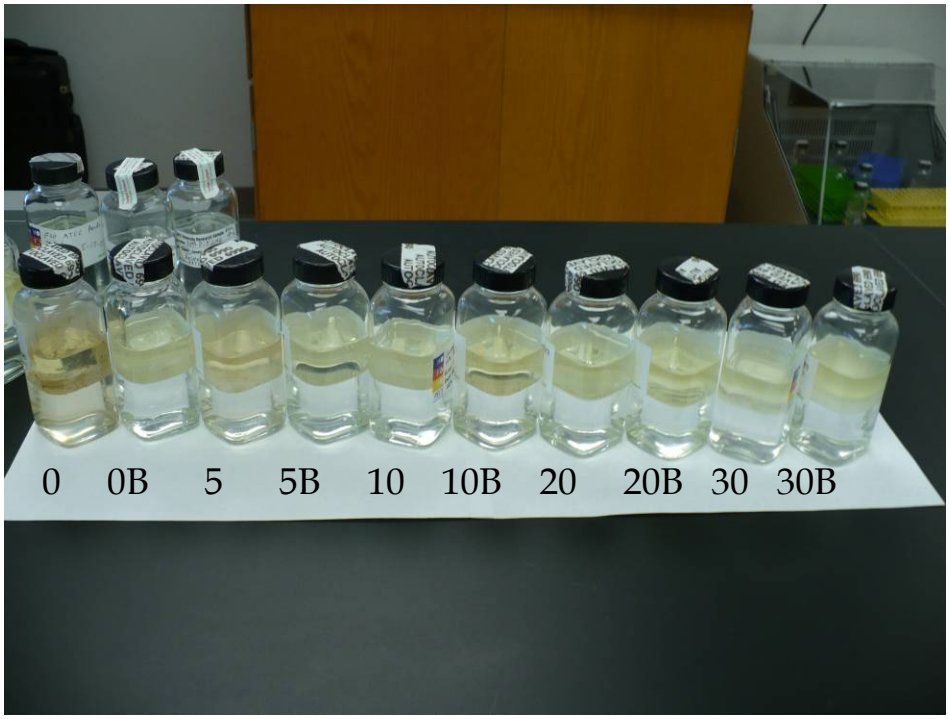

Figure 21. Field consortia following day 46 of test. DiEGME concentrations are: 0, 5, 10, 20, and 30% by volume in water phase, with each next to its respective blank.

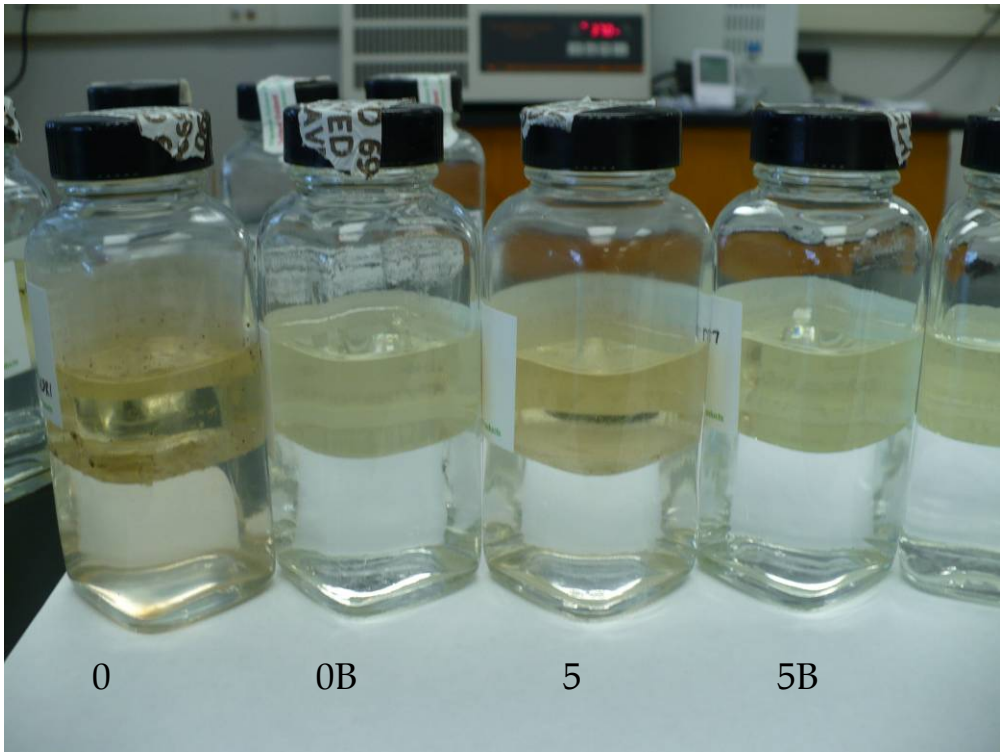

Figure 22. Field consortia following day 46 of test. DiEGME concentrations are: 0 and 5% by volume in water phase, with each next to its respective blank.

Figures 21 and 22 show that the field consortia test setups are visually similar to the ATCC consortia setups. Like the ATCC setups, the field consortia setups show some cloudiness in the water layer, as well as particulates and cloudiness in the fuel layer for both the 0 and 5% DiEGME levels. A biofilm was present at the 0% and 5% levels, but not at the higher concentration levels. No visual differences were apparent for the 10% level and above test setups, compared to the ATCC consortia.

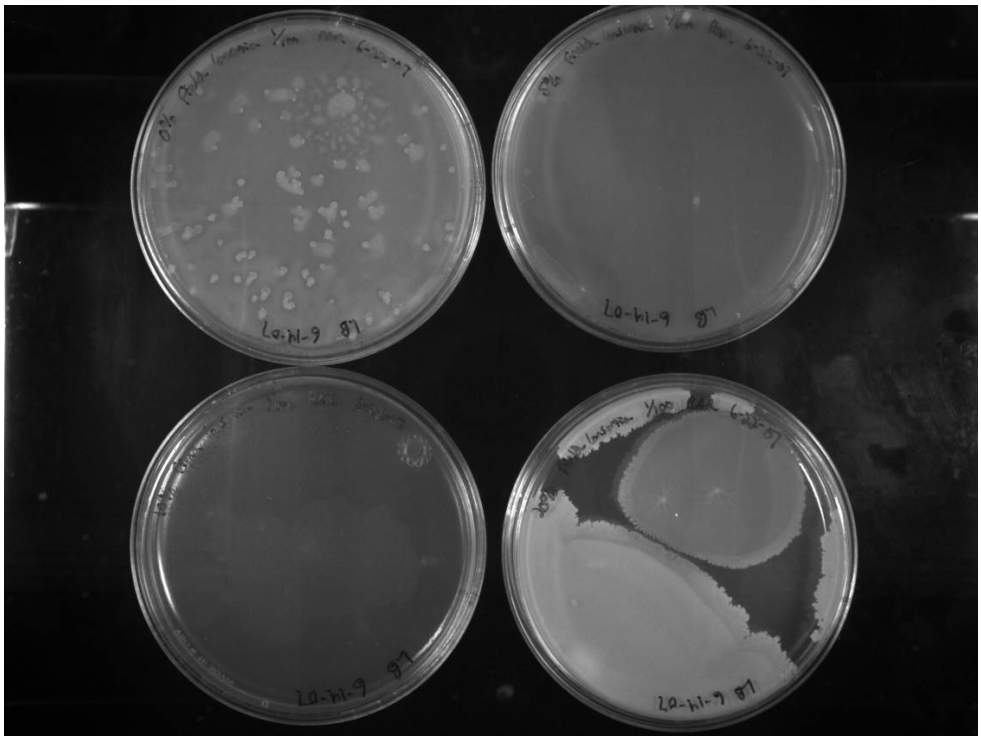

Figure 23. Six field consortia at day 8. DiEGME concentrations are: 0 (upper left), 5 (upper right), 10 (lower left), and 20% (lower right), 1/100 dilution. All plates are swarming with growth.

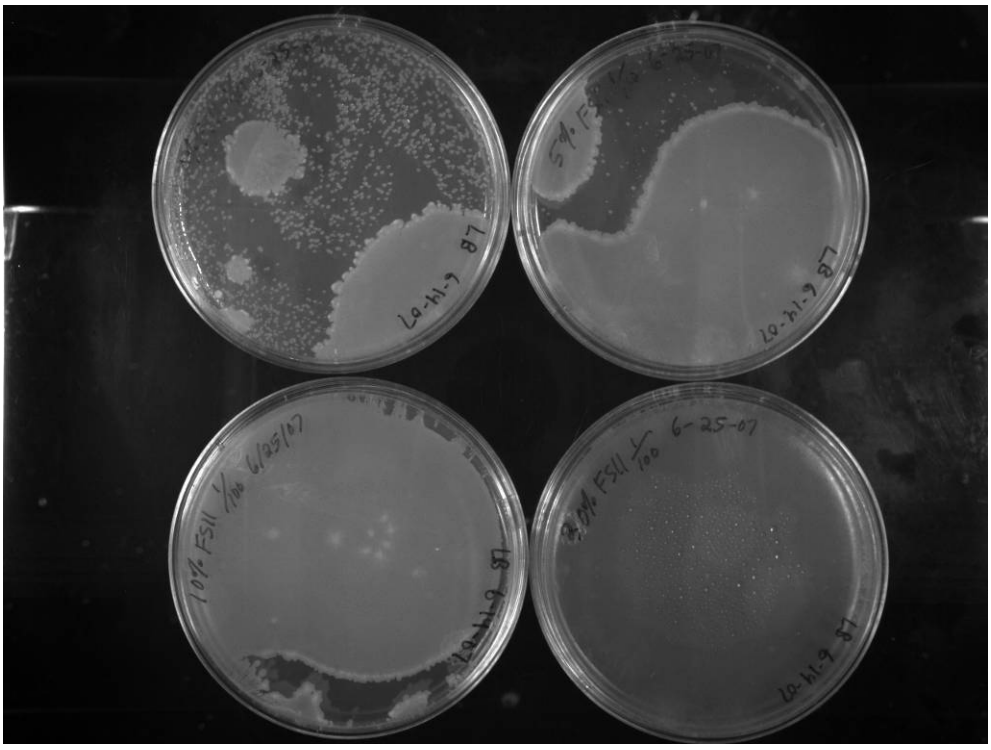

Figure 24. Six field consortia at day 11. DiEGME concentrations are: 0 (upper left), 5 (upper right),10 (lower left), 20% DiEGME (lower right), 1:100 dilution. All plates are swarming with growth.

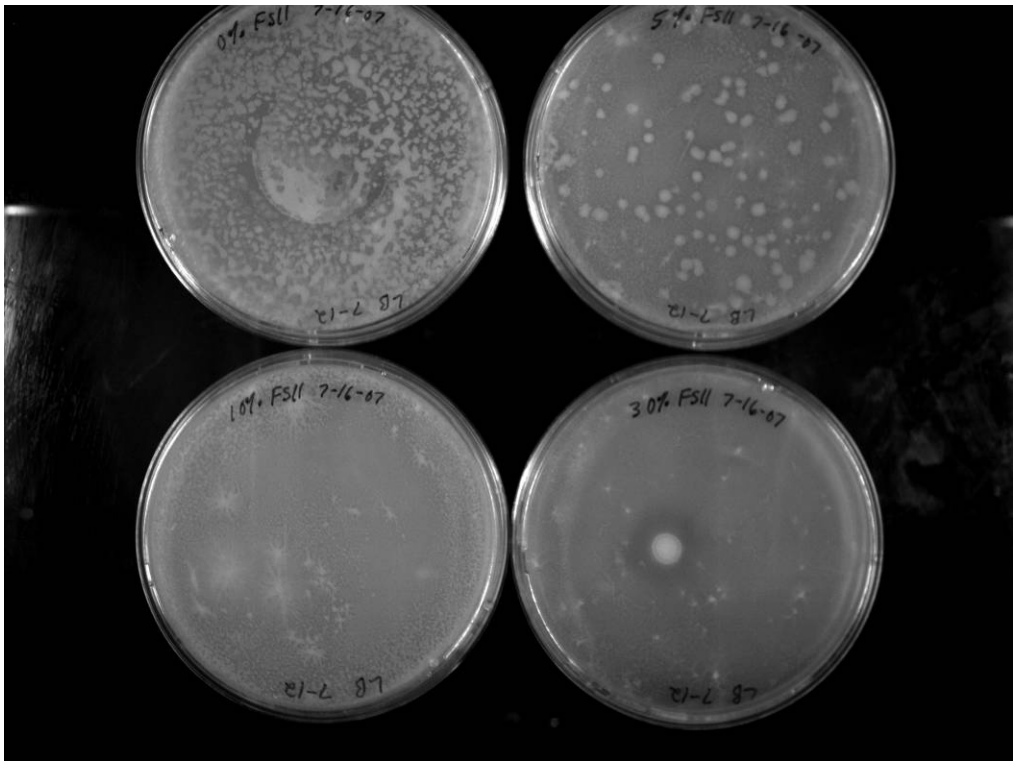

Figure 25. Six field consortia at day 32. DiEGME concentrations are: 0 (upper left), 5 (upper right),10 (lower left), and 30% DiEGME (lower right).

Figures 23-25 show agar plates at several points during the 46 day test period. Some differences can be seen between the ATCC microorganisms' colony growth and that of the field consortia. For example, the field consortia growth is not completely halted over the test period as is the ATCC microorganism growth. However, the field consortia growth is also significantly reduced at DiEGME levels of 10% and above.

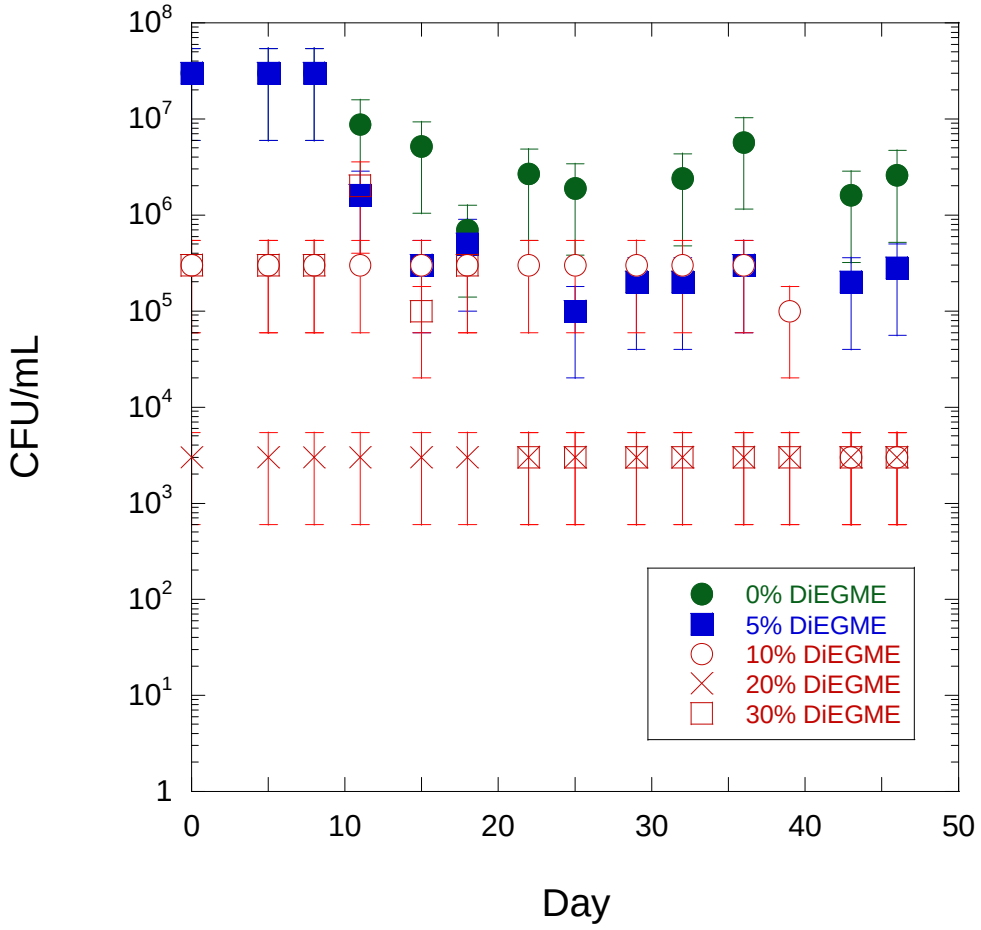

Figure 26. Semi-log plot of six field consortia colony counts over a 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

Figure 26 shows that, unlike the ATCC consortia, the mixed field consortia were viable throughout the test period. However, the presence of DiEGME clearly reduced the amount of growth at each test period. It seems possible that differences in response to FSII are the result of genetic mutations within the field microbes over time. These mutations can occur through several pathways. For example, it could be that the microbial mutations have occurred in response to low levels of some man-made chemical, such as DiEGME, in their environment. The ATCC microorganisms were collected from the environment prior to the widespread usage of DiEGME in aviation fuel, and it is possible they were not able to reap the benefits of 20 years or more of environmental adaptation undergone by at least some members of the field consortia.

Another possibility is that resistance to DiEGME was transferred to the field microbes by other microbes that became resistant following their own exposure to the substance—meaning the field microbes in this study did *not* require direct exposure to DiEGME, but only the sharing of genetic material (plasmid swapping) with microbes that were exposed, to develop mutations. Yet another possibility is that field microbial resistance was developed without prior additive exposure via hypermutation or some other adaptation mechanism. (26) Although it is clear that the low levels of DiEGME in this study did not kill all six field microorganisms, the difference between the 0% DiEGME samples and the other DiEGME samples was dramatic, indicating the continuing usefulness of a biocidal/biostatic fuel additive for control of microbial growth. Less growth was apparent for both ATCC and field consortia microorganisms at the 5% DiEGME by volume in water. For the ATCC

microorganisms, the ATCC *Pseudomonas* was subdued at the 5% level, but there was still a significant increase in growth at the 5% level over the 46 day test period for the ATCC *Candida* and the ATCC *Cladosporium*. Some reduction in growth was noted at the 5% FSII level for the field consortia—approximately an order of magnitude—but the overall growth at 5% was still fairly high. At the 10% DiEGME level and above, however, dramatic downward shifts in growth begin to occur for the ATCC microorganisms, whose growth is eliminated completely in fewer than 15 days. For the field microorganisms, there is also a downward shift in growth at the 10% level, though it took longer for the decrease in growth to occur. For the ATCC microorganisms, the 15-30% DiEGME levels resulted in faster kills than the lower levels. For the field microorganisms, the higher levels did not result in complete kills, but microbial growth was decreased by almost three orders of magnitude, in some cases immediately. Generally, it can be said that, regardless of whether the microorganisms are lab cultured or from the field, DiEGME still has a beneficial biocidal/biostatic effect. DiEGME also seems to impede the development of harmful biofilms. The current study suggests that a DiEGME level of 10% by volume in the aqueous phase at minimum (0.01-0.02% ppm added to the fuel) is required to have a significant effect on microbial growth. These levels are considerably lower than the current typical concentration of approximately 30-50% by volume in the aqueous phase (0.1-0.15% ppm added to the fuel). An important question that still needs to be explored, however, is which of the field microorganisms continues to survive at all DiEGME levels tested here.

Table 1. Diversity of colony types vs. percent volume of DiEGME in aqueous phase

Day	Number of Colony Types-0% DiEGME	5%	10%	20%	30%	Total Number of Types
0	3	3	3	3	3	3
4	2	1	1	1	1	2
8	1	1	1	1	1	2
11	2	1	0	1	1	2
15	2	2	1	1	1	2
18	1	1	1	1	1	1
22	2	2	2	2	2	2
25	1	1	1	1	1	2
29	1	1	1	1	1	1
32	1	1	2	1	1	2
36	1	1	1	1	1	1
39	2	1	1	1	1	2
43	2	1	1	1	1	2
46	1	1	1	1	1	1

As shown in Table 1, the results of the platings suggest that the mixed consortia decreased in diversity with increasing DiEGME concentration, which suggests that, even in the field, there may only be a small percentage of microbes that can survive DiEGME.

In this study, preliminary results suggested that perhaps only one or two of the six field microbes persisted throughout the test period. Additional work described below addresses the identification of these exceptional microbes. Analysis of the genetic differences which allow them to survive will be the subject of future work.

4.3 Additional Field Microorganisms Consortia Test at Higher DiEGME Concentrations

Because two of the six field microbes persisted in the original study using 0-30% DiEGME by volume in aqueous phase, an additional study was conducted to determine whether DiEGME was more effective on these microbes at typical DiEGME concentrations seen in the field, which can be ~30-60% in the aqueous phase. The additional study was conducted with the same methodology utilized in section 3.4, except the concentrations tested were: 30, 40, 50, and 60% DiEGME by volume in the aqueous phase, which corresponds to ~0.05-0.15% in the fuel phase. Figures 27 and 28 show liquid test setups following the 46 day test. Figures 29-31 show colony plating results at several points during the test. Figure 32 illustrates colony counts throughout the 46 day test period.

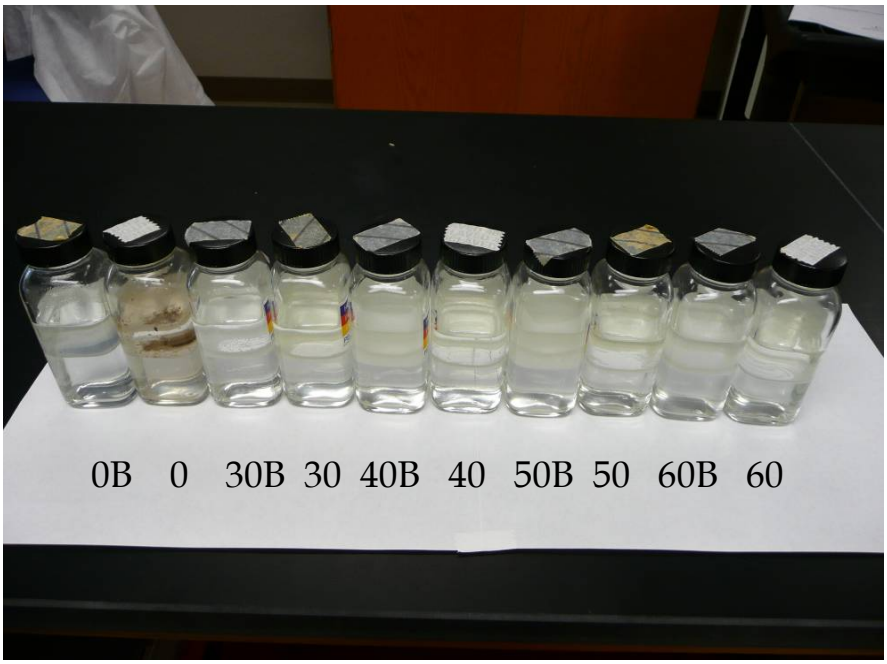

Figure 27. Additional field consortia liquid setups after 46 days. Concentrations are 0, 30, 40, 50, and 60% DiEGME in the aqueous phase. Each is shown with its respective blank.

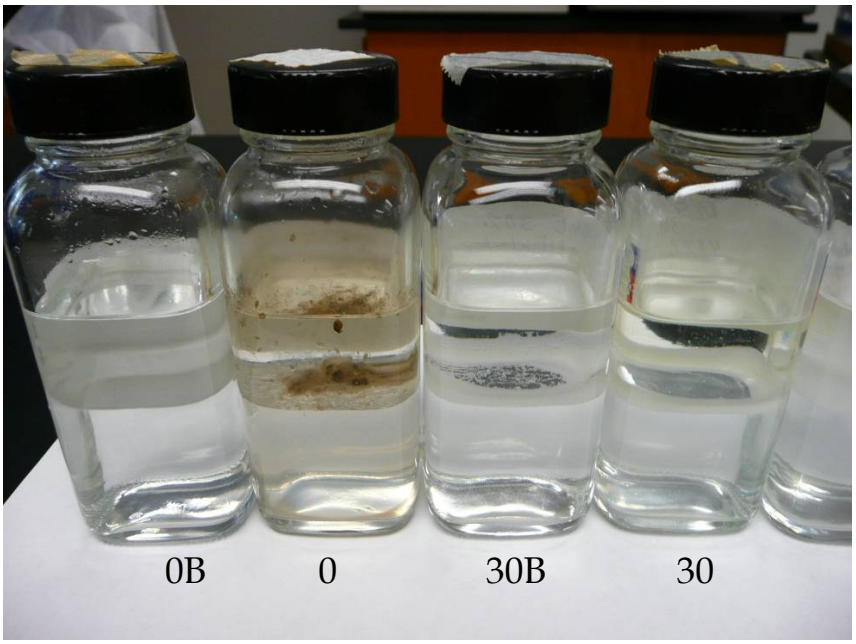

Figure 28. Additional field consortia on day 46. Closeup of 0 and 30% DiEGME with their blanks.

Figures 27 and 28 indicate that substantial growth is only present in the 0% DiEGME liquid setup. A brown biofilm in the hydrocarbon phase is readily apparent, as is significant cloudiness in the aqueous phase. Both suggest significant microbial contamination. The corresponding blank has no growth nor obvious cloudiness. The 30-60% DiEGME liquid setups appear very similar to their blanks, although the blanks appear to be slightly less cloudy in the aqueous phase. At these concentrations of DiEGME, emulsions are persistent and occur for both inoculated and blank liquid setups.

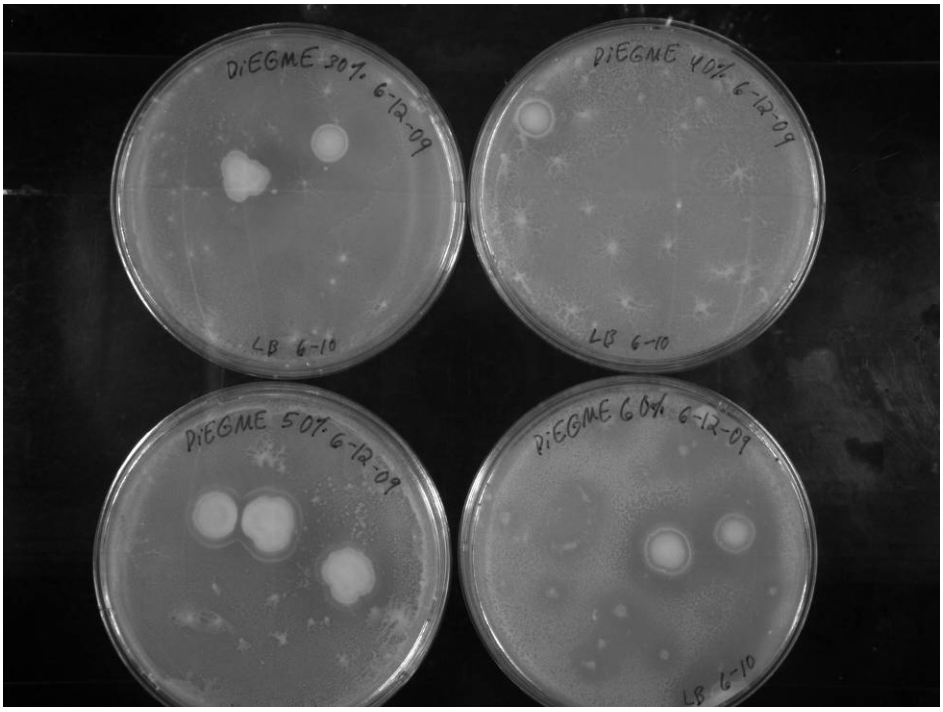

Figure 29. Additional field consortia study on day 4. DiEGME concentrations of 30, 40, 50, and 60% in the aqueous phase are shown.

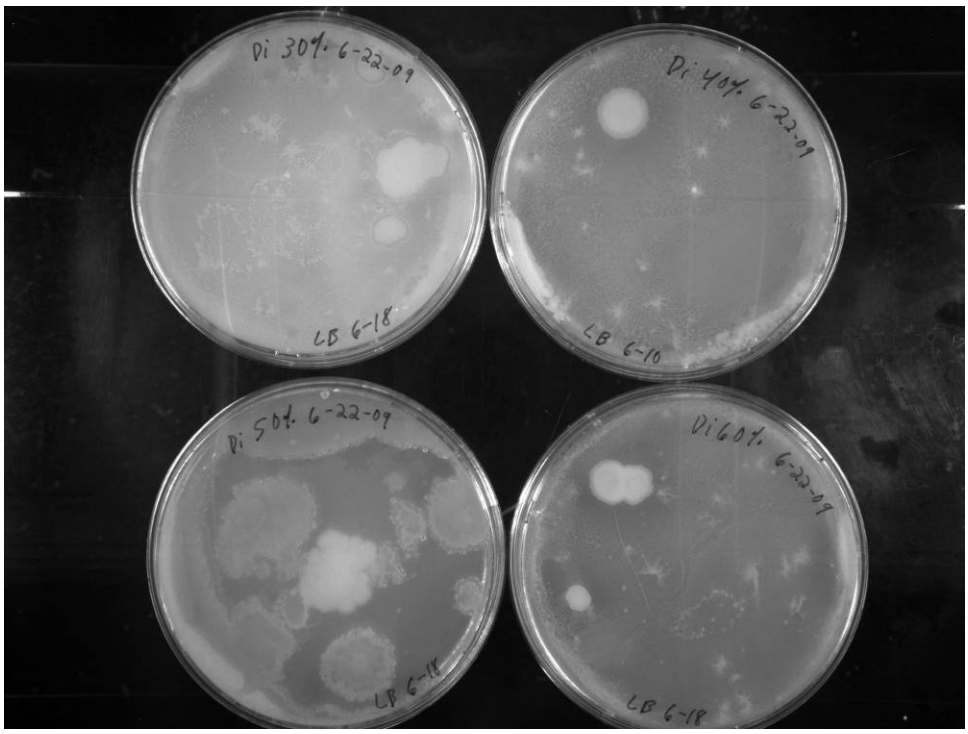

Figure 30. Additional field consortia study on day 14. DiEGME concentrations of 30, 40, 50, and 60% in the aqueous phase are shown.

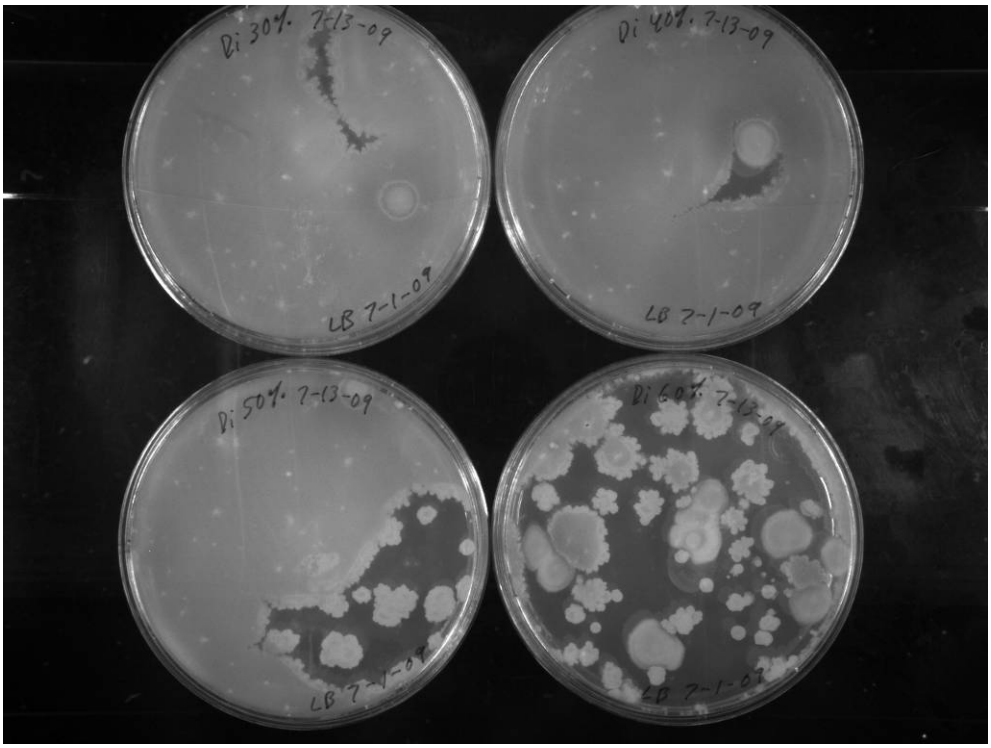

Figure 31. Additional field consortia study on day 35. DiEGME concentrations of 30, 40, 50, and 60% in the aqueous phase are shown.

The colony plate results shown in figures 29-31 indicate healthy microbial growth at all test points shown, although it does appear that the amount of colonies is somewhat decreased in the second half of the test duration. Morphological evaluation of these colonies suggests that the *Bacillus* and *Clostridium* obtained from the field are able to survive in the presence of DiEGME, even at concentrations of 30-60% DiEGME in the aqueous phase.

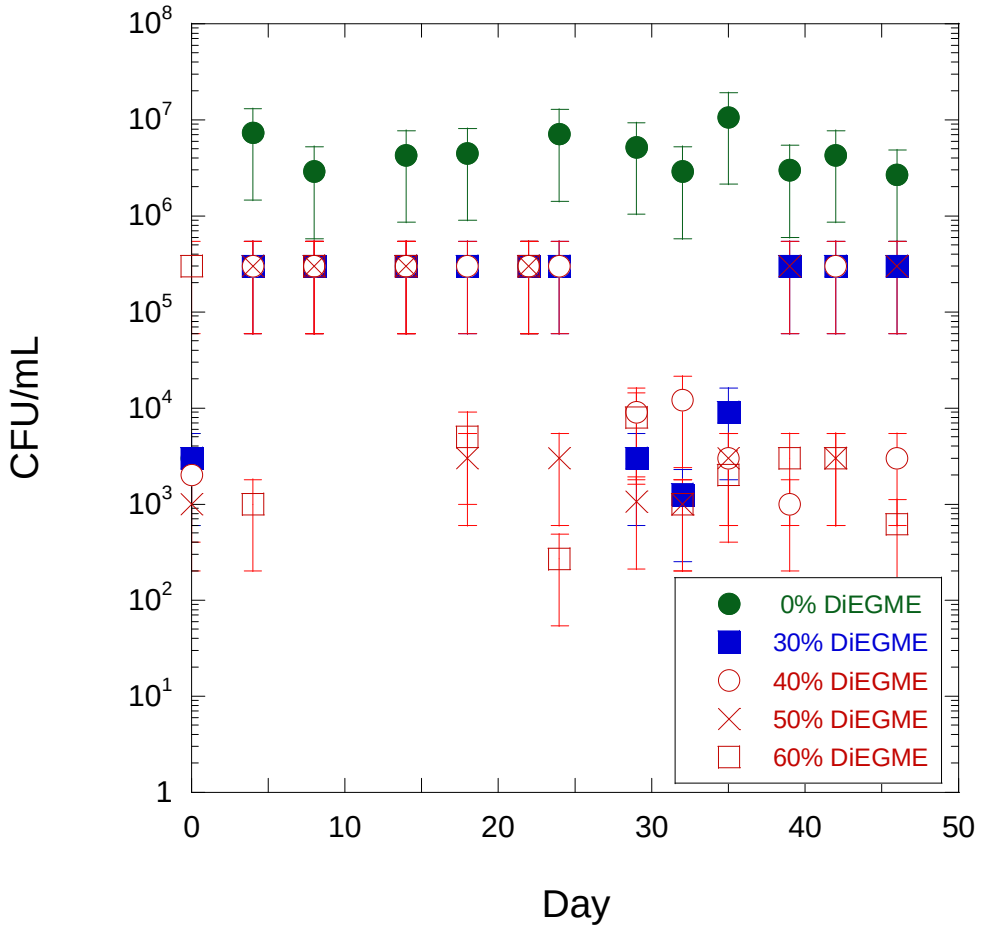

Figure 32. Semi-log plot of additional field consortia test colony counts over a 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

Figure 32 illustrates that, over the 46 day test period, the presence of DiEGME at 30-60% decreases the amount of field consortia colonies present, but does not eliminate growth completely. Furthermore, decreases in numbers are not necessarily obtained with an increase in DiEGME concentration. Rather, the threshold seen for the original study—DiEGME present at 10% or greater in the aqueous phase—seems to provide approximately the same protection as the higher DiEGME concentrations currently present in the field.

5. Conclusions

This study of the biocidal/biostatic effects of DiEGME at reduced levels was conducted in support of a larger program aimed at reducing current FSII levels for purposes of cost reduction and reduction of harmful DiEGME side effects, such as topcoat peeling in the B-52. This study explored the potential biological impact of lowering the current DiEGME concentration from ~0.05-0.15% in the fuel phase (30-60% by volume in the aqueous phase at ambient temperature) to ~0.0-0.05% in the fuel phase (0-30% by volume in the aqueous phase at ambient temperature). Although other studies have been conducted to determine if DiEGME was effective at low levels, none were designed in an attempt to reflect the most common microbial contaminants across the current air fleet. In the current study, where such field microorganisms were included, in addition to lab cultured ATCC microbes, it was found that DiEGME levels of ~0.01-0.02% in the fuel phase (10% by volume in the aqueous phase at ambient temperature) or greater were sufficient to eliminate the ATCC microbes and significantly reduce the growth of the field microbes tested. Additional testing revealed that, of the six field microbes, two persisted even at current DiEGME levels, although the overall consortia growth level was reduced. The two persistent bacteria were *Bacillus* and *Clostridium*. In addition, the presence of DiEGME at ~0.01-0.02% or greater was shown to inhibit the formation of harmful biofilms. The results of this study suggest that levels of ~0.01-0.02% DiEGME and above (10% by volume and above in the aqueous phase at ambient temperature) still act beneficially to control microbial growth in aircraft fuel systems.

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Appendix

Chemical Analysis of Selected Samples

Polar species levels were analyzed for ATCC consortia samples following 46 day testing. Polars are trace levels of oxygen, nitrogen, and/or sulfur-containing heteroatomic species. The purpose of the polars analysis was to determine if it was possible to detect microbial activity via a previously reported HPLC polar species detection and quantitation method developed for jet fuel analysis (29). Many polar species commonly exist in jet fuel and have been linked to thermal and/or storage instability. Low polar levels typically indicate high thermal stability, and high polar levels typically indicate low thermal stability. Substituted phenols, for example, are very common polar species in aviation fuel. Many of the products bacteria create while utilizing hydrocarbon fuel as an energy source are also various types of polar oxygenates, including aromatic, and were therefore hypothesized to be detectable by HPLC-UV. Fuel layer samples were taken from neat 4877, 4877 with consortia--0% DiEGME, 4877 with consortia—5% DiEGME, 4877 with consortia—10% DiEGME, and 4877 with consortia—30% DiEGME. Changes in polar species levels typically indicate chemical changes in the fuel, and in this trial experiment, polar species measurements were taken to determine if it was possible to detect microbial action. Measurements were taken following the 46 day test. The low measurement threshold is 23 mg/L, and the error in measurement is expected to be $\pm$ 1.3%. The results are shown below:

Table 2. Polars content of neat 4877 fuel vs. 4877 fuel with ATCC consortia

	4877 Neat	**4877** w/consortia 0% FSII	**4877** w/consortia 5% FSII	**4877** w/consortia 10% FSII	**4877** w/consortia 30% FSII
Polars by HPLC (mg/L)	**30**	**100**	**190**	**90**	**90**

The polars levels clearly rose following the addition of the consortia in all cases. However, it is not clear why the 5% polars level would be so much higher than the 0% level. It is possible that the presence of FSII results in additional detectable products. It is also not clear why the 10% and 30% have such high polar levels relative to the 0% and 5%, samples which experience microbial growth for a considerably longer period. A more complete study, following the polars levels throughout the test period, would probably clarify the issue.

In addition, samples from the 0 and 5% were analyzed by SPE, followed by GC-MS. Although most of the chemical constituents found were indistinguishable from those normally seen in jet fuel, a couple seemed to definitely be the result of microbial activity: 2,5 cyclohexadiene-1,4 dione (also known as quinone) and 1-dodecanol. These were large peaks in the 5% sample. More research is needed to determine if these compounds are always indicative of microbial growth, especially in the presence of DiEGME. If that is the case, this test might point the way to a sensitive analysis technique for estimating microbial activity in jet fuel samples.

LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

ACRONYM	***DESCRIPTION***
AFPET	Air Force Petroleum Office
ATCC	American Type Culture Collection
CFU	Colony Forming Units
DSCR	Defense Supply Center Richmond
DiEGME	Diethylene Glycol Monomethyl Ether
EPS	Extracellular Polymeric Substances
FSII	Fuel System Icing Inhibitor
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LB	Luria-Bertani
MIC	Microbially Induced Corrosion
SSIS	Small-Scale Icing Simulator
TNTC	Too Numerous To Count
USAF	United States Air Force
USN	United States Navy